

SUBLETHAL EFFECTS OF DIETARY SELENIUM EXPOSURE ON JUVENILE FISHES

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By D. Landon McPhee

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ABSTRACT

Selenium (Se) is known to cause chronic toxicity in aquatic species. In particular, dietary exposure of fish to selenomethionine (SeMet), the primary form of Se in the diet, is of concern. Previous studies reported that chronic exposure to elevated dietary SeMet altered swimming performance, aerobic metabolism, and energy and endocrine homeostasis in adult fish. However, little is known about the direct effects of dietary SeMet exposure in juvenile fish. Therefore, the overall objective of this thesis was to investigate sublethal pathophysiological effects of subchronic dietary SeMet exposure in two juvenile fish species, fathead minnow (*Pimephales promelas*) and rainbow trout (*Oncorhynchus mykiss*). In the first experiment, 20 days post hatch (dph) juvenile fathead minnow were exposed to different measured concentrations (2.8, 5.4, 9.9, 26.5 $\mu\text{g Se/g dry mass [dm]}$) of Se in food in the form of SeMet for 60 days. In the second experiment, 14 dph juvenile rainbow trout were exposed for 37 days to different measured concentrations (1.0, 4.1, 11.2, 26.1 $\mu\text{g Se/g dm}$) of Se in food in the form of SeMet. Following exposure, samples were collected for Se analysis and fish were subjected to a swimming performance challenge to assess critical swim speed (U_{crit}), tail beat frequency and tail beat amplitude, oxygen consumption (MO_2), cost of transport (COT), standard metabolic rate (SMR), active metabolic rate (AMR), and factorial aerobic scope (F-AS).

Dietary SeMet exposure impaired swimming ability in both fathead minnow and rainbow trout. Juvenile fathead minnow showed alterations in aerobic metabolism with increased MO_2 , COT and AMR at the 9.9 and 26.5 $\mu\text{g Se/g diets}$, while dietary SeMet exposure did not appear to affect aerobic metabolism in juvenile rainbow trout. After

swim performance experiments, swam fish were considered fatigued and metabolic and energy storage endpoints were compared to non-swam (non-fatigued) fish.

Energy storage capacity was measured via whole body (fathead minnow) and liver and muscle (rainbow trout) triglyceride and glycogen concentrations. For fathead minnow, triglyceride concentrations in non-swam fish were significantly elevated in the 5.4 $\mu\text{g Se/g}$ group relative to controls, and swam fish had significantly lower whole body triglycerides than non-swam fish. All non-swam SeMet exposure groups had significantly decreased whole body glycogen concentrations compared to controls while the 5.4 and 26.5 $\mu\text{g Se/g}$ exposure groups had significantly greater whole body glycogen concentrations in swam versus non-swam fish.

In juvenile rainbow trout, liver triglyceride concentrations were significantly lower in all SeMet exposed groups compared to controls in non-swam fish. Swimming decreased liver and muscle triglycerides in the control and 11.2 $\mu\text{g Se/g}$ treatment groups. Liver glycogen concentrations were greater in swam trout in the 4.1 $\mu\text{g Se/g}$ exposure group. Muscle glycogen concentrations in non-swam fish, were significantly decreased in the 4.1 and 11.2 $\mu\text{g Se/g}$ exposed groups compared to controls, while muscle glycogen in swam fish was unaffected by dietary SeMet exposure. For the swim status factor, muscle glycogen concentrations were significantly greater in swam versus non-swam trout in all treatment groups.

Therefore, dietary SeMet exposure caused impaired swimming performance and metabolic alterations in both juvenile fathead minnow and juvenile rainbow trout. Species differences were apparent, especially in the patterns of altered energy status between swam and non-swam fish exposed to Se. Overall, the pathophysiological

implications of these sublethal effects are unclear, but suggest that dietary SeMet exposure may negatively influence juvenile fish survivability in natural habitats.

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LIST OF ABBREVIATIONS

$\mu\text{g/g}$	Micrograms per gram
μg	Microgram
$\mu\text{g/L}$	Micrograms per litre
μm	Micrometre
ANOVA	Analysis of variance
ATRF	Aquatic Toxicology Research Centre
ATSDR	Agency for Toxic Substances and Disease Registry
cm	Centimetre
d	Day
dm	Dry mass
EC10	Effective concentration in 10 percent of a population
g	Gram
ICP-MS	Inductively coupled plasma-mass spectrometry
kg	Kilogram
L	Litre
LOD	Limit of detection
m	Metre
mg	Milligram
mg/kg	Milligrams per kilogram
mg/L	Milligrams per litre
mL	Millilitre
mm	Millimetre
MS-222	Tricaine methanesulfonate
n	Number of samples
NSERC	Natural Sciences and Engineering Research Council of Canada
O_2	Oxygen
$^{\circ}\text{C}$	Degrees centigrade
Se	Selenium
Se^0	Solid elemental selenium
Se^{-2}	Inorganic selenide
Se^{+4}	Selenite
Se^{+6}	Selenate
SEM	Standard error of the mean
SeMet	Selenomethionine
t_i	Increment time length in Critical swimming speed test
t_f	The duration of the last velocity increment until fatigue in Critical swimming speed test
U_{crit}	Critical swimming speed
V_i	Velocity increase per time increment in Critical swimming speed test
V_p	Final velocity swam in Critical swimming speed test
XAS	X-ray absorption spectroscopy
XANES	X-ray absorption near-edge spectroscopy

PREFACE

Chapter 1 of this thesis is a general introduction and Chapters 2 and 3 are organized as manuscripts for publication in scientific journals. Thus, there is some repetition of introductions, materials and methods throughout each chapter. Chapter 2 has been submitted to *Aquatic Toxicology* and Chapter 3 will be submitted to *Comparative Physiology and Biochemistry Part C*.

CHAPTER 1

1.0 GENERAL INTRODUCTION

1.1 Selenium in the environment

Selenium (Se) is an element belonging to the VIA group on the periodic table of elements and shares similar properties with elements such as sulphur. It is classified as a non-metal and has several metalloid properties (Young et al., 2010). Selenium is naturally occurring with a wide global distribution. Redistribution through environmental compartments occurs from a variety of natural and anthropogenic sources. Geological sources of Se include phosphate rocks, black shale, and limestone (Maher et al., 2010). Natural processes that contribute to the redistribution of Se in the environment include volcanic activity, weathering of rocks, wildfires, and volatilization from plants and water bodies (Maher et al., 2010). While natural cycling is important to global changes in Se, the largest sources are generally related to anthropogenic activities (Presser et al., 1990; Maher et al., 2010). Metal mining and milling operations, oil refining, fossil fuel production, industrial manufacturing, and agricultural processes are some of the major sources of Se contamination in aquatic ecosystems (Lemly, 2004; Maher et al., 2010). Selenium can be transported to aquatic environments in water or air, with water acting as the primary delivery method of Se from anthropogenic sources (Maher et al., 2010).

1.2 Selenium speciation, cycling, uptake, bioaccumulation and biomagnification

Selenium exists in a number of different oxidation states in aquatic environments: selenate (Se^{+6}), selenite (Se^{+4}), elemental selenium (Se^0), and selenide (Se^{-2}) (USEPA, 2004). The elemental form of aqueous Se is rarely found as Se most commonly enters

aquatic environments from point and non-point sources as inorganic selenite and selenate. The cycling of Se is a complex and dynamic process governed by biological, chemical, and physical processes. The availability of Se species is highly variable and dependent on environmental conditions such as oxygen content, organic material, microbial content, and water mixing and flow patterns (Lemly, 1997a, 1999; ATSDR, 2003; Simmons and Wallschläger, 2005).

The trophic transfer of Se is a threat to aquatic ecosystems due to its high bioavailability. The key route of Se uptake in fish is via the diet. Primary producers such as algae, bacteria and fungi facilitate the uptake of Se from the water column. The water soluble oxyanions are fixed in the tissues of these primary producers and biotransformed into the organoselenide compounds selenocysteine and selenomethionine (SeMet) (Fan et al., 2002). These organic Se compounds are readily bioavailable compared to inorganic forms (Bowie et al., 1996). Once ingested by consumer organisms, these selenoamino acids are incorporated into proteins which are subsequently transferred to other organisms upon ingestion. As a result, higher trophic level organisms may accumulate higher levels of Se than their surrounding environment. Microorganisms are tolerant of Se exposure and act as vectors that deliver high dietary Se from lower trophic organisms to fish. Therefore, in aquatic environments, relatively low aqueous Se concentrations (1–5 µg Se/ L) can lead to Se bioaccumulation in the food chain (Stewart et al., 2010).

Selenomethionine (SeMet) is the dominant form of Se in the diet and is efficiently bioaccumulated in aquatic food webs (Fan et al., 2002; Young et al., 2010). Because SeMet cannot be synthesized by vertebrates, excess levels not metabolized will be taken up into tissues that have a high rate of protein synthesis including the liver, gonads, kidneys, and skeletal muscle. In these tissues excess SeMet can be incorporated into proteins

in place of methionine (Reilly, 1996; ATSDR, 2003; Bakke et al., 2010). Selenomethionine is arbitrarily substituted for methionine during protein synthesis in a random, dose-dependent manner. On the contrary, the incorporation of selenocysteine into proteins is highly regulated at the ribosomal level by selenocysteinyl – tRNA and is associated with Se essentiality, and thus, has been recognized as the 21st amino acid (Stadtman, 1996; Schrauzer, 2000; Janz et al., 2010).

1.3 Biochemical and toxicological aspects of Se

1.3.1 Selenium essentiality

Selenium is recognized as an essential micronutrient required by animals to maintain normal physiological function (Schwarz and Foltz, 1957). Vertebrates incorporate Se into a number of different selenoproteins in the form of selenocysteine (Kryukov et al., 2003; Lu and Holmgren, 2009). In particular, fish are thought to have one of the largest known selenoproteomes among animals with 30 to 37 proteins requiring Se (Lobanov et al., 2009). Selenoproteins are known to be involved in diverse physiological functions such as protection from oxidative stress, thyroid hormone activation and inactivation, selenoprotein synthesis and Se transport (Reilly, 1996; Behne et al., 2000; Kryukov et al., 2003). Selenocysteine is found at the active site of antioxidant enzymes such as glutathione peroxidase (Reddy and Massaro, 1983; Reilly, 1996). Glutathione peroxidases are important antioxidant enzymes in fish and mammals that use glutathione as a cofactor to scavenge reactive oxygen species (ROS) and reduce intracellular peroxides. Selenium is also necessary for normal function of thioredoxin reductase, an enzyme involved in DNA synthesis, oxidative stress and protein repair (Beckett and Arthur, 2005).

1.3.2 Selenium toxicity

Although Se is a required micronutrient, there is a very narrow range between essentiality and toxicity in fish (Janz et al., 2010). Dietary concentrations of 0.1 to 0.5 µg Se/g dry mass (dm) are required for normal selenoprotein function in fish. However, dietary concentrations greater than 3.0 µg Se/g dm can potentially result in increased Se accumulation and toxicity (Lemly, 1997b). Fish are among the most sensitive organisms to Se exposure (Janz et al., 2010; Janz, 2012). In particular, larval and juvenile fish display a number of developmental abnormalities when exposed chronically to elevated Se concentrations (Lemly, 1997b; Kennedy et al., 2000; Holm et al., 2005; Muscatello et al., 2006; Muscatello and Janz, 2009). The maternal transfer of Se into eggs and subsequent yolk assimilation is the key mechanism responsible for toxicosis.

Selenomethionine is incorporated into the yolk protein precursor vitellogenin in place of methionine. It is the transport and uptake of vitellogenin containing SeMet from the liver to the oocytes that ultimately determines the Se dose received by eggs (Janz et al., 2010; Janz, 2012). As a result, several permanent developmental anomalies such as skeletal, craniofacial, and fin malformations and edema are exhibited. These deformities form the basis of a diagnostic suite characteristic of Se exposure in fish (Janz et al., 2010). These overt morphological pathologies have the potential to be lethal to fish and greatly affect their recruitment into fish populations (Woock et al., 1987; Janz, 2012). The comparative sensitivity of fish to Se induced reproductive toxicity (i.e. deformities) is relatively narrow. For example, the span of EC10 (or equivalent threshold) values for eight fish species ranged from 17 to 24 mg Se/kg dm in eggs, separating the most sensitive species (cutthroat trout [*Oncorhynchus clarki*]) by only a factor of 1.4 from the most tolerant

species (fathead minnow [*Pimephales promelas*]) (Janz et al., 2010). Due to the well-defined examples of embryo and larval toxicity of Se to fish, the earlier life stages are also likely to be the most sensitive to direct dietary Se exposure (Teh et al., 2002; Muscatello et al., 2006; Tashjian et al., 2006).

1.3.3 Mechanisms of action

Selenium is thought to exert its toxic effects through two main mechanisms of action. The first has to do with the similarities between sulphur and Se. Selenium toxicity can be attributed to the shared chemical characteristics with sulphur and its ability to substitute for that element during protein synthesis (Maier and Knight, 1994; Lemly, 1997b). When Se erroneously substitutes for sulphur in cysteine and methionine, disulfide linkages are disrupted and the tertiary structure of proteins is altered, potentially resulting in impaired protein and enzyme function (Diplock and Hoekstra, 1976; Reddy and Massaro, 1983; Sunde, 1984).

The second mechanism of action involves toxic effects resulting from Se-induced oxidative stress. Oxidative stress occurs when the production of reactive oxygen species (ROS) overcomes the cellular defence mechanisms, resulting in toxic effects that include lipid peroxidation, DNA and protein oxidation, and cellular apoptosis. The metabolism of Se results in intermediate compounds that are redox reactive and capable of generating superoxide and hydrogen peroxide (Spallholz, 1994). In addition, SeMet is catabolised to the reactive intermediate methylselenol, further contributing to oxidative stress (Wang et al., 2002; Spallholz and Hoffman, 2002; Palace et al., 2004; Spallholz et al., 2004; Misra et al., 2010). Palace et al. (2004) hypothesized that the occurrence of pericardial and yolk sac edema in response to dietary SeMet exposure were the result of an increase in

superoxide anion in rainbow trout (*Oncorhynchus mykiss*) embryos via generation of methylselenol intermediate. *In vitro* selenite exposure generated ROS in rainbow trout hepatocytes (Misra and Niyogi, 2009). In addition, SeMet exposure to Japanese medaka (*Oryzias latipes*) embryos led to a consumption of intracellular glutathione, resulting in oxidative stress (Lavado et al., 2012). Further investigation is required to gain a complete understanding of the mechanism of oxidative stress in fish as a result of Se exposure.

1.4 Swimming performance in fish

Swimming is integral to fish survival and fitness and is paramount to many fish behaviours. Fish are dependent on swimming for predator avoidance, food acquisition, schooling, migration and mating. Any impairment of swimming performance could have effects at the organismal, population, and ecosystem level (Beaumont et al., 1995; Drucker, 1996; Plaut, 2001; Scott and Sloman, 2004). Therefore, swim performance assessment can be a valuable and ecologically relevant tool in the investigation of contaminant stress and toxicity. Various test protocols have been used to examine swimming performance. Most swim tests involve placing fish in a chamber to swim against a current for determined amounts of time. Fish swimming can be characterized by three prominent modes: sustained, burst and prolonged (Beamish, 1978).

1.4.1 Swim performance classifications

Sustained swimming involves fish maintaining locomotion without fatigue for long periods of time, often greater than 240 minutes but occasionally for days or months (Beamish, 1978; Hammer, 1995). Sustained swimming performance is fuelled aerobically with balanced metabolic demands and waste removal (Jones, 1982; Hammer,

1995). The investigation of sustained swimming ability is resource intensive and has therefore received less interest than other swimming types. However, it can provide important information on the state of aerobic metabolism, bioenergetic and hormonal status in fish subjected to sustained exercise (Hammer, 1995).

Burst swimming speed can only be maintained for short periods (less than 20 seconds) and is fuelled anaerobically (Beamish, 1978; Jones, 1982; Hammer, 1995). Exhaustion from burst swimming occurs due to depletion of cellular energy stores or accumulated waste products of anaerobic metabolism (pyruvate and lactate) that inhibit muscle function (Black et al., 1962; Brett, 1964). Burst swimming is integral to fishes ability to maintain an efficient escape response from predators and acquire food sources and therefore is an ecologically relevant measure of fitness. Typically, it is measured by applying an external stimulus to fish maintained at low water speeds and recording the rapid escape response (Domenici and Blake, 1997).

Prolonged swimming consists of swimming ability in the time periods between burst and sustained swimming, typically lasting between 2 and 200 minutes and ending in exhaustion (Hammer, 1995; Plaut, 2001). Like sustained swimming, it is fuelled by aerobic metabolism (Jones, 1982; Hammer, 1995; Plaut, 2001). Prolonged swimming ability involves aspects of both sustained and burst swimming, therefore making it an attractive endpoint of study in fish swim performance (Brett, 1964; Hammer, 1995). In natural habitats, field studies of prolonged swimming prove challenging due to its difficulty in separating from sustained swimming behaviour. Alternately, laboratory based swim performance tests were developed to accurately assess fatigue related to prolonged swimming ability (Beamish, 1978; Plaut, 2001). Some studies have also concluded that prolonged swimming is influenced by behaviour in addition to physiology (Nelson, 1990).

In swim performance tests, fish are placed in a tunnel supplied with a flow of oxygenated water, and must swim at specific speeds for periods of time. Two commonly utilized tests are fixed velocity and incremental velocity tests (Hammer, 1995). In fixed velocity tests, fish are subjected to a single flow rate that is steadily increased until a maximum velocity is reached. The time until fish fatigue at maximum velocity is recorded. Incremental velocity tests involve the evaluation of the ability of a fish swimming against a current that is increased in a time-dependent stepwise manner until the onset of fatigue (Hammer, 1995). Incremental velocity tests are more common than fixed velocity due to their requirements of fewer test species and shorter experimental time relative to fixed velocity tests (Hammer, 1995).

The speed at which a fish fatigues is called the Critical Swim Speed (U_{crit}) (Brett, 1964) and is calculated as:

$$U_{crit} = V_p + ((t_f/t_i) \times V_i).$$

Where:

V_p is the maximum velocity step the fish swam, V_i is the velocity increment (cm/sec), t_f is the elapsed time from the velocity increase to fatigue, and t_i is the time between velocity increase steps (Brett, 1964; Hammer, 1995).

Critical swimming speed is an organism-level indication of the state of the physiological and biochemical fitness of fish. Thus, swim performance analysis can provide an ecologically relevant evaluation of sublethal effects of contaminant exposure in addition to more commonly studied biochemical, toxicological, and morphological endpoints (Kolok, 2001). Environmental conditions may not accurately be reflected by standard toxicity endpoints. Fishes survival is greatly dependent on predator avoidance,

food acquisition, schooling, migration and mating. Any impairment of swimming performance could have effects at the organismal, population, and ecosystem level. Therefore, swim performance assessment can be a valuable tool in the investigation of contaminant stress and toxicity.

1.4.2 Respirometry

The availability of oxygen to fish is an important factor influencing swimming performance. The metabolic processes in fish depend on efficient respiration to deliver adequate oxygen uptake and waste removal. Oxygen is delivered via the gills to the systemic circulation, where it is then distributed for aerobic metabolic processes. Carbon dioxide and nitrogenous wastes are also excreted via the gills. Increased demanding activity results in an oxygen-debt, and anaerobic metabolism occurs until oxygen reduction causes fatigue. Because U_{crit} is a measure of overall swimming ability, it takes into account both aerobic and anaerobic metabolism. Swimming respirometry is often used in tandem with U_{crit} to gain an understanding of the oxygen consumption (MO_2) of fish. Oxygen consumption (MO_2) is a measure of metabolic activity of fish, and alterations in MO_2 are indicators of stress commonly related to changes in aerobic metabolism (Brett, 1972; Watenpaugh and Beiting, 1985). During swimming performance tests, aerobic metabolism is thought to be responsible for 80% of U_{crit} (Webb, 1971; Hammer, 1995; Moyes and West, 1995). Therefore, reduced oxygen availability may hinder aerobic metabolism in tissues and significantly impair swimming performance. Temperature can strongly influence aerobic function in fish with potential repercussions on swimming ability in fish. Water temperature influences oxygen solubility with increased temperatures associated with reduced oxygen saturation and decreased water temperature

associated with increased oxygen saturation. Therefore differences in water temperature could be responsible for variations in MO_2 during swimming (Lee, 2003; Farrell et al., 2008).

Oxygen consumption can also be used to investigate a number of other useful endpoints to assess aerobic metabolism and swimming performance. These include cost of transport (COT), the standard metabolic rate (SMR), the active metabolic rate (AMR), and the factorial aerobic scope (Videler, 1993; Shingles et al., 2001; Lee, 2003; Claireaux et al., 2006; Norin and Malte, 2011; Clark et al., 2013). Cost of transport represents the energy required by a fish to move a unit of distance and is an index of overall swimming efficiency (Videler, 1993). The SMR represents the minimal metabolic rate for maintenance activity and increased SMR can indicate greater energy requirements due to the biotransformation and excretion of toxicants and/or the repair of toxicant induced damage (Priede, 1985; Rowe et al., 2001; Norin and Malte, 2011). The AMR is the oxygen consumption at maximum exercise and is the upper boundary for aerobic metabolism (Norin and Malte, 2011). Factorial aerobic scope (F-AS) is an indicator of proportional increases in oxygen consumption rates in fish from rest to maximum exercise and is the ratio of AMR to SMR (Clark et al., 2013).

1.4.3 Swim motion

Fish swimming is defined by the transfer of momentum from the fish to the surrounding water (Sfakiotakis et al., 1999). The propulsive movement in fish depends on the caudal fin and whole body motion (Webb, 1975). Most fish species use lateral body undulations running from head to tail to propel swim motion (Wardle et al., 1995). There are three main classifications based on these propulsive motions. Anguilliform

swimmers use whole-body undulations to propel forward motion (Sfakiotakis et al., 1999). Conversely, thunniform swimmers depend on the lateral motion of the caudal fin for propulsion. Examples of thunniform fish include the species of tuna and mackerel. Subcarangioform swim motion is comparable to anguilliform, except the lateral undulations are limited to the anterior of the body, and increase posteriorly (Sfakiotakis et al., 1999). Examples of subcarangioform swimmers include salmonid and cyprinid species (Kaufmann, 1990). Swim motion can be evaluated by video recording movement in a swim tunnel and comparing the movements at different time points during the performance test.

Tail beat frequency and tail beat amplitude are two endpoints commonly used to characterize swim motion. Tail beat frequency is the number of completed beat cycles of the fish tail over a period of time. This endpoint can provide important additional information when assessed along with U_{crit} due to the positive relationship between swimming speed and tail beat frequency in carangiform and subcarangiform swimmers (Bainbridge, 1958; Ohlberger et al., 2007). In addition, tail beat frequency can also be used as a predictive tool for U_{crit} and oxygen consumption during steady swimming in some fish (Steinhausen et al., 2005). Tail beat amplitude can also be measured along with U_{crit} to supplement the swim performance testing. It is based on the lateral movement of the fish tail as it oscillates left and right from the midline during steady swimming and measures the maximum tail stroke distance from the tip of the tail to the longitudinal body line. Taken together, tail beat frequency and amplitude can be useful in assessing muscle function of fish during swim performance analyses (Videler and Wardle, 1991; Herbing, 2002; Tudorache et al., 2010). Toxicant-induced morphological

changes can negatively impact swim motion and thus alter U_{crit} and general swim performance. Defects to the fin or spine can drastically affect the ability of larval fish to avoid predation and feed, and thus potentially result in death. However, fish with more subtle deformities may persist into juvenile and adult life stages (Lemly, 1993, 1998).

1.5 The physiological stress response

Fish possess an integrated stress response that is necessary for re-establishing homeostasis upon exposure to stressors (Wendelaar Bonga, 1997; Sapolsky et al., 2000). A suite of primary, secondary, and tertiary responses to stress are commonly found in vertebrates. The initiation of the primary stress response results in neuroendocrine stimulation of the chromaffin cells and the hypothalamic-pituitary-interrenal (HPI) axis, resulting in the release of catecholamines and glucocorticoid hormones respectively (Jobling, 1994; Wiseman et al., 2011b). Corticosteroids are released from the head kidney in response to the presence of adrenocorticotrophic hormone (ACTH) in the bloodstream. In teleost fish, cortisol is the key glucocorticoid (Jobling, 1994). The release of catecholamines results in shorter-term metabolic action such as the stimulation of glucose production by glycogenolysis, whereas cortisol produces longer lasting homeostatic effects including the stimulation of gluconeogenesis (Wendelaar Bonga, 1997; Mommsen et al., 1999). This neuroendocrine system involvement can then trigger a host of secondary metabolic effects. The secondary phase of the stress response is characterized by the mobilization of energy stores. Liver glycogenolysis and gluconeogenesis, as well as mobilization of lipids from tissues are triggered through the action of the catecholamines (Reid et al., 1998). Cortisol targets the liver, resulting in increases in hepatic glycogen mobilization and systemic glucose concentration. In

addition, plasma lactate is also elevated in the blood during the secondary stress response (Mommsen et al., 1999). The tertiary response is characterized by chronic exposure to a stressor resulting in pathological changes that can ultimately lead to decreased reproduction, growth, and pathogen immunity (Jobling, 1994). The physiological stress response is a highly integrated process that has a number of implications on the bioenergetics of the organism.

1.6 Energy storage and metabolism

Triglycerides and glycogen are the key forms of energy storage in fish (Dangé, 1986; Tocher, 2003; Thomas and Janz, 2011). Fish use these energy stores for general functions such as locomotion, growth, and reproduction (Thomas and Janz, 2011). Therefore, alterations to stored energy levels can have significant effects on fish health. Exposure to environmental contaminants has been shown to impact triglyceride and glycogen concentrations in fish (Levesque et al., 2002; Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Goertzen et al., 2011; Thomas and Janz, 2011).

Triglycerides are the key form of lipid storage in fish (Jobling, 1994). Triglycerides are primarily stored in the liver, muscle and visceral tissue. Under increased energy demand, these lipid stores can be mobilized and subsequently transported in the systemic circulation as free fatty acids. As a result, lipid reserves are generally depleted during periods of stress. Lipids are also important in maintaining normal physiological function in fish. They play key roles in growth and survival, as well as provide the main fuel source for prolonged swimming via the aerobic process of triglyceride β -oxidation (Webb, 1971; Moyes and West, 1995; Pratt and Fox, 2002; Biro et al., 2004; Goertzen et al., 2011). The first 80% of the U_{crit} test is fueled by aerobic

catabolism of triglyceride stores in muscle tissue (Hammer, 1995; Moyes and West, 1995).

Glycogen is the primary form of stored carbohydrate in fish and is stored mainly in the liver and muscle tissue (Jobling, 1994). As mentioned above, glycogen is important for maintaining homeostasis during the physiological stress response. Both increases and decreases in glycogenolysis can occur as a result of toxicant exposure and can alter the concentrations of glycogen (Giesy et al., 1988). In general, the increased energy demand due to stress results in the depletion of glycogen stores (Ansaldo et al., 2006; Lin et al., 2011). Intramuscular glycogen levels are also important in the swimming ability of fish. The breakdown of glycogen in muscle provides the necessary energy required for burst swimming (Webb, 1971; Hammer, 1995). For U_{crit} tests investigating prolonged swimming performance, glycogen acts as a fuel source via anaerobic glycolysis at swim speeds beyond 80% U_{crit} to exhaustion. Therefore, the determination of glycogen concentration can be a useful tool to assess the exposure to toxicants as well as the overall health and metabolic capacity of fish.

1.7 Effects of selenium exposure on swimming performance and energy homeostasis

1.7.1 Effects of Se on swimming performance

Alterations to energy homeostasis in fish have potential negative impacts to fish health. If these effects persist in the natural environment, populations of fish species could be at risk. As mentioned previously, fish use glycogen and triglycerides to fuel swimming. Impaired swimming performance may alter food acquisition, prey avoidance and reproduction, and such alterations could ultimately impact fitness and survival of

wild fish. The critical swimming speed (U_{crit}) is a common measure used to assess swimming performance in fish (Brett, 1964). Adult zebrafish (*Danio rerio*) exposed to dietary SeMet showed a significant reduction in U_{crit} along with increased levels of cortisol, glycogen, and triglycerides (Thomas and Janz, 2011). Because of the increases in stored energy, one would expect a strong performance in the swimming challenge. However, the decrease in U_{crit} indicates some inability of the fish to access its stored energy reserves. In addition, fathead minnow exposed to metal mine effluent resulted in a decrease in U_{crit} (Goertzen et al., 2011). These fish also had reduced activity of citrate synthase, indicating the possible inhibition of a key process in the catabolism of glycogen and therefore were not able to access these energy stores for swimming. Abnormal swimming behaviour has also been reported in juvenile bluegill (*Lepomis macrochirus*) exposed to waterborne selenite and selenate (Cleveland et al., 1993). Other alterations to swimming ability such as reduced activity, swimming belly up, and confinement to tank bottom have been shown in juvenile sacramento splittail (*Pogonichthys macrolepidotus*) and white sturgeon (*Acipenser transmontanus*) exposed to dietary SeMet (Teh et al., 2004; Tashjian et al., 2006). These findings show that Se has pronounced effects on the swimming ability of fish. In particular, the accessibility of energy reserves appears to be inhibited. Further studies are needed to delineate the mechanisms of the effect of Se on swimming ability. The alterations to the physiological stress response and subsequent utilization of energy have impacts on the swimming performance of fish, potentially threatening fitness and survival in the wild.

1.7.2 Effects of Se on stress response

Selenium exposure has effects on the physiological stress response in fish. In particular, Se causes alterations in the concentrations of cortisol. Rainbow trout exposed to water-borne selenite in both acute (94 hour) and subchronic (30 day) studies have shown a significant increase in plasma cortisol concentrations (Miller et al., 2007). The acute selenite exposure resulted in a greater plasma cortisol release than a subchronic exposure, demonstrating that acute exposure to waterborne selenite is more stressful to rainbow trout than a 30-day exposure (Miller et al., 2007). In addition, white sucker (*Catostomus commersoni*) from Se-impacted streams had increased amounts of cortisol in blood plasma compared to fish from a reference site (Miller et al., 2009). While waterborne Se is an important exposure pathway to fish, the dietary exposure to selenomethionine represents the major route of Se exposure in aquatic environments (Janz, 2012). Indeed, chronic dietary exposure to selenomethionine elevates the physiological stress response in fish. Adult zebrafish fed a concentration of 26.6 µg Se/g dm for 90 days showed elevated levels of whole body cortisol production (Thomas and Janz, 2011). Dietary SeMet exposure also has the potential to attenuate the stress response in fish. In a study by (Wiseman et al., 2011b), rainbow trout exposed to an environmentally relevant concentration of dietary SeMet demonstrated increased concentrations of cortisol consistent with other studies (Miller et al., 2007; Thomas and Janz, 2011). However, fish subjected to a secondary handling stressor showed a clear attenuation of the cortisol response (Wiseman et al., 2011b). Therefore, dietary selenomethionine exposure has the potential to significantly impede the ability of fish to mount a successful stress response when faced with multiple stressors. While the

mechanism of the attenuated cortisol response remains unknown, the reduced concentrations of cortisol in blood plasma from trout exposed to SeMet and a subsequent handling stressor most likely is the result of increased metabolism of cortisol (Wiseman et al., 2011b). In contrast to the general trend of increased cortisol levels in Se exposed fish, the impairment of secretion of cortisol has also been reported. The adrenocortical cells of rainbow trout and brook trout (*Salvelinus fontinalis*) showed an impairment of cortisol secretion upon exposure to selenite (Miller and Hontela, 2011). Interestingly, only selenite and not SeMet caused this impairment (Miller and Hontela, 2011). In addition to the effects of Se on cortisol, the potential exists for effects further downstream in the physiological stress response.

1.7.3 Effects of Se on energetics

Selenium exposure has been demonstrated to cause alterations in the amounts of stored energy in fish. Zebrafish chronically exposed to dietary SeMet showed a significant increase in both whole body glycogen and triglycerides (Thomas and Janz, 2011; Thomas et al., 2013). Similarly, rainbow trout chronically exposed to dietary SeMet also demonstrated a significant increase in glycogen and triglycerides in muscle tissue (Wiseman et al., 2011b). Both of these studies also reported increased cortisol concentrations in exposed fish, suggesting some impairment of the secondary stress response. As mentioned above, the secondary phase of the stress response involves the mobilization of energy stores, resulting in increased plasma glucose and lactate (Mommensen et al., 1999). However in the previous study, rainbow trout showed no significant differences in plasma glucose and lactate concentrations between controls and SeMet exposed groups (Wiseman et al., 2011b). An acute selenite exposure study of

rainbow trout resulted in an increase in plasma cortisol along with a subsequent increase in plasma glucose levels (Miller et al., 2007). The differences between these two examples suggest that the metabolism of glycogen and triglycerides could be impacted. Changes to enzyme activities involved in energy metabolism including phosphofructokinase, lactate dehydrogenase, creatine kinase and citrate synthase have been observed in fish after exposure to environmental contaminants (Almeida et al., 2001; Konradt and Braunbeck, 2001). Citrate synthase in particular is a rate-limiting mitochondrial enzyme involved in the citric acid cycle, and thus, is an indicator of tissue aerobic capacity and glycolysis (Rajotte and Couture, 2002). Any impairment of this enzyme could indicate inhibited metabolism of energy stores. Juvenile fathead minnow exposed to metal-mine effluent known to contain Se showed a reduction in the activity of citrate synthase (Goertzen et al., 2011). In addition, fish collected from Se-impacted sites have shown increased concentrations of glycogen and triglyceride stores (Bennett and Janz, 2007; Driedger et al., 2010; Kelly and Janz, 2008).

1.8 Fish species of interest

Fathead minnow are a cyprinid species that is the most widely used small fish model for regulatory ecotoxicology and aquatic toxicology research in North America (Ankley and Villeneuve, 2006). Its distribution is relatively broad throughout North America in both lentic and lotic systems. Fathead minnows have a well-characterized reproductive/development cycle, as well as the ability to tolerate a wide range of water chemistry conditions and temperatures, making it an attractive species for laboratory based research (Ankley and Villeneuve, 2006). In the wild, fathead minnows are

opportunistic omnivores but in laboratory settings are commonly maintained on brine shrimp (genus *Artemia*), blood-worms (family *Chironomidae*), or flaked food. The size, combined with the well-established developmental stages of this species, make it an attractive model for investigating swimming performance and the pathophysiological effects of dietary SeMet exposure.

Like fathead minnow, rainbow trout have a wide distribution and are found throughout North America (Behnke and Tomelleri, 2002). This member of the Salmonidae family has been widely utilized for research in the fields of toxicology, carcinogenesis, comparative immunology, disease ecology, and nutrition (Thorgaard et al., 2002). Rainbow trout are typically found at temperatures ranging from 6°C to 23°C, with an optimal temperature of 12°C. In addition, rainbow trout are the most frequently studied fish species when it comes to exercise physiology with more information known about this species than any other (Milligan, 1996). They are also known for swimming great distances, swimming upstream against substantial currents and jumping up waterfalls during reproductive migration (Webb, 1994). Thus, rainbow trout make an excellent test species for the investigation of the effects of dietary SeMet exposure on swimming performance, aerobic metabolism and energy homeostasis.

1.9 Research objectives and hypotheses

The overall goal of this thesis was to investigate the effects of subchronic dietary SeMet exposure on juvenile fishes. To accomplish this, a number of biochemical, physiological, behavioural, and morphological endpoints were determined. Critical swim speed and swim motion were used to evaluate swimming performance. Swimming trials

were based on the method previously described by Brett (1964). Swim tunnel respirometry was used to measure aerobic metabolism during swim testing. Whole body (fathead minnow) and tissue (rainbow trout; liver and muscle) concentrations of triglycerides and glycogen were measured in swam and non-swam fish to evaluate energy storage capacity. Whole body cortisol was also determined to investigate the physiological stress response.

1.9.1 Objectives

- 1) Assess swim performance, aerobic metabolism, energy utilization and storage capacity, the physiological stress response, and behavioural effects in juvenile fathead minnow exposed to a range of dietary SeMet concentrations for 60 days.
- 2) Assess swim performance, aerobic metabolism, energy utilization and storage capacity, the physiological stress response, and behavioural effects in juvenile rainbow trout exposed to a range of dietary concentrations SeMet for 37 days.

1.9.2 Hypotheses

- 1) Subchronic exposure to dietary SeMet would impair swimming performance in juvenile fathead minnow and juvenile rainbow trout.
- 2) Impaired aerobic metabolism would occur in juvenile fathead minnow and juvenile rainbow trout in the form of altered oxygen consumption and metabolic rates during swimming performance trials following a subchronic dietary SeMet exposure.
- 3) Subchronic exposure to dietary SeMet would alter energy homeostasis by increasing energy stores and cortisol concentrations in juvenile fathead minnow and juvenile rainbow trout.

CHAPTER 2

2.0 DIETARY SELENOMETHIONINE EXPOSURE ALTERS SWIMMING PERFORMANCE, METABOLIC CAPACITY AND ENERGY HOMEOSTASIS IN JUVENILE FATHEAD MINNOW

This investigation was performed as the first of two studies in this thesis and was undertaken to determine the effects of dietary Se exposure on juvenile fish. Juvenile fathead minnow were exposed to elevated dietary concentrations of Se in the form of SeMet for 60 days. After exposure, samples were collected for Se analysis and fish were subjected to a swimming performance challenge to assess critical swim speed (U_{crit}), tail beat frequency and tail beat amplitude, oxygen consumption (MO_2), cost of transport (COT), standard metabolic rate (SMR), active metabolic rate (AMR), and factorial aerobic scope (F-AS). Energy storage capacity and the physiological stress response were measured via whole body triglyceride and glycogen concentrations and whole body cortisol concentrations, respectively. The results of this study suggest that exposure to environmentally relevant concentrations of dietary SeMet impairs swimming performance, aerobic capacity, and energy homeostasis, potentially impacting survivability of juvenile fish in Se impacted aquatic ecosystems.

2.1 Introduction

Selenium is an essential trace element required by all vertebrate species to maintain normal physiological function. Vertebrates, and other organisms, incorporate Se into a number of different selenoproteins as selenocysteine (Kryukov et al., 2003; Lu and Holmgren, 2009). Fish express 30 to 37 selenoproteins, and are believed to possess the largest known selenoproteomes among animals (Lobanov et al., 2009). Proteins containing selenocysteine are known to be involved in diverse physiological functions such as protection from oxidative stress, thyroid hormone production, and selenoprotein synthesis (Kryukov et al., 2003). Although Se is a required micronutrient, there is a very narrow range between essentiality and toxicity in fish (Janz et al., 2010). Dietary levels of 0.1 to 0.5 $\mu\text{g Se/g dry mass (dm)}$ are sufficient for normal selenoprotein function in fish. However, dietary concentrations greater than 3.0 $\mu\text{g Se/g dm}$ can potentially result in increased Se accumulation and toxicity (Lemly, 1997b).

Selenium occurs naturally in the environment in soils, sediments, black shale, coal, and phosphate rocks with anthropogenic activities such as metal mining and milling operations, fossil fuel production, industrial manufacturing, and agricultural processes acting as major sources of Se contamination in aquatic ecosystems (Maher et al., 2010). Selenium enters aquatic environments in its soluble ionic forms of selenite and selenate. Primary producers and microorganisms, such as algae, accumulate this inorganic Se and biotransform it into organoselenides, primarily selenocysteine and selenomethionine (Fan et al., 2002). Once ingested by consumer organisms, these selenoamino acids are incorporated into proteins which are subsequently transferred to other organisms upon ingestion. As a result, organic forms of Se are much more likely to bioaccumulate at

higher trophic levels than inorganic forms of Se. Primary producers are tolerant of Se exposure and act as vectors that deliver high dietary Se from lower trophic organisms to fish, which are among the most sensitive organisms (Janz et al., 2010; Janz, 2012). Therefore, in aquatic environments, relatively low aqueous Se concentrations (1–5 µg Se/ L) can lead to toxicity in fish via bioaccumulation in their prey (Muscatello et al., 2006; Stewart et al., 2010; Janz et al., 2010; Janz, 2012). Selenomethionine (SeMet) is the dominant form of Se in the diet (Fan et al., 2002). Due to its similarity in molecular structure, SeMet is arbitrarily substituted for methionine during protein synthesis in a random, dose-dependent manner (Janz, 2012).

Selenium exposure is known to cause severe developmental abnormalities in larval and juvenile fish (Lemly, 1997b; Muscatello et al., 2006; Muscatello and Janz, 2009). The maternal transfer of Se into eggs and subsequent yolk assimilation by fish larvae is a key mechanism responsible for toxicosis. In adult female fish, SeMet is incorporated into the yolk protein precursor vitellogenin in place of methionine. The transport and uptake of vitellogenin containing SeMet from the adult liver to oocytes ultimately determines the Se dose received by eggs (Janz et al., 2010; Janz, 2012). The utilization of SeMet and potentially other organoselenium species in the yolk of the developing embryos can result in a host of skeletal, craniofacial and fin malformations and edema that are characteristic of exposure (Holm et al., 2005; Muscatello et al., 2006; Janz, 2012). In addition to larval deformities, a number of sublethal effects are also known to occur in adult and juvenile fish exposed to Se in the diet. For example, chronic exposure to environmentally relevant dietary concentrations of SeMet in adult zebrafish (*Danio rerio*) resulted in alterations to swimming ability, aerobic metabolism, and energy

storage capacity (Thomas and Janz, 2011; Thomas et al., 2013). An attenuation of the physiological stress response and altered reproductive endocrine function has been demonstrated in fish exposed to dietary SeMet (Wiseman et al., 2011a, 2011b). While sublethal effects of dietary Se in adult fish have been demonstrated, less is known on the sublethal effects in juvenile fish. Due to the well-defined examples of embryo and larval toxicity of Se to fish, the earlier life stages are also likely to be the most sensitive to direct dietary Se exposure (Teh et al., 2002; Muscatello et al., 2006; Tashjian et al., 2006). Sublethal effects in juvenile fish may persist into adult life stages, potentially affecting recruitment and population sustainability. Therefore, investigation of sublethal toxicities of dietary Se exposure in juvenile fish remains a pressing research need.

Swim performance analysis provides an ecologically relevant evaluation of sublethal effects of contaminant exposure in addition to more commonly studied lethal endpoints (Little and Finger, 1990; Kolok, 2001). Fish survival is greatly dependent on a number of swimming centric behaviours such as predator avoidance, food acquisition, schooling, migration and mating. Therefore, any impairment of swimming performance could have effects at the organismal, population, and ecosystem level (Scott and Sloman, 2004; Thomas and Janz, 2011). Critical swimming speed (U_{crit}) is a commonly used method of assessing swim performance in fish (Brett, 1964). The U_{crit} test uses incremental increases in velocity until exhaustion to determine the maximum swimming velocity prior to fatigue. Because aerobic metabolism is responsible for up to 80% of U_{crit} , this test is a standard measure of aerobic performance (Webb, 1971). Another indicator of fish swimming performance and behaviour includes two characteristics of

swim motion, tail beat frequency and tail beat amplitude (Bainbridge, 1958; Steinhausen et al., 2005; Ohlberger et al., 2007; Thomas and Janz, 2011).

Swimming respirometry is often used in tandem with U_{crit} . Oxygen consumption (MO_2) is a measure of metabolic activity of fish, and alterations in MO_2 are indicators of stress commonly related to changes in aerobic metabolism (Brett, 1972; Watenpaugh and Beiting, 1985). Oxygen consumption can also be used to investigate a number of other useful endpoints of metabolism. These include cost of transport (COT), which is a measure of the energetic cost of swimming, the standard metabolic rate (SMR), which is the minimal metabolic rate of unfed fish, the active metabolic rate (AMR), which is the metabolic rate at maximum sustainable velocity during the U_{crit} test, and the factorial aerobic scope, which is the ratio of AMR to SMR (Videler, 1993; Shingles et al., 2001; Lee, 2003; Claireaux et al., 2006; Norin and Malte, 2011; Clark et al., 2013). Swim performance in combination with MO_2 thus provides sensitive measures of sublethal exposure to Se. For example, adult zebrafish exposed to aqueous selenite exhibited a decrease in U_{crit} (Massé et al., 2013), while adult zebrafish exposed to dietary SeMet experienced a decrease in U_{crit} and increased MO_2 (Thomas and Janz, 2011; Thomas et al., 2013).

Swimming performance and MO_2 are closely related to the physiological stress response and energy homeostasis. Cortisol is the major corticosteroid hormone in teleost fish and is essential to maintaining normal energy metabolism and homeostasis (Mommsen et al., 1999). During the physiological stress response, cortisol is released from the interrenal cells or head kidney. Plasma cortisol in large bodied fishes and whole body cortisol in small bodied fishes are useful measurements of physiological stress

(Wendelaar Bonga, 1997; Ramsay et al., 2006). Adult zebrafish exposed to a SeMet spiked diet exhibited elevated cortisol concentrations (Thomas and Janz, 2011). In addition, both an increase in plasma cortisol concentrations and an attenuation of the cortisol response has also been shown in rainbow trout (*Oncorhynchus mykiss*) exposed chronically to an SeMet augmented diet (Wiseman et al., 2011b). The production of cortisol during the physiological stress response is closely tied to energy metabolism and homeostasis. During periods of stress, cortisol targets the liver to mobilize triglyceride and glycogen stores. Triglycerides and glycogen are the main forms of stored energy in fish and are both used to fuel swimming (Tocher, 2003; Thomas et al., 2013). Routine, sustained and prolonged swimming is fuelled aerobically by the catabolism of triglycerides, while burst swimming is mainly fuelled by the anaerobic catabolism of glycogen (Hammer, 1995; Moyes and West, 1995). During the U_{crit} test, triglycerides act as the energy source for the contraction of slow oxidative red muscle fibres up to approximately 80% U_{crit} (Hammer, 1995; Moyes and West, 1995). Beyond 80% U_{crit} , glycogenolysis is initiated and glycogen acts as the primary energy source to power the action of fast glycolytic white muscle fibres (Webb, 1971; Hammer, 1995; Moyes and West, 1995). Elevated Se exposures have been reported to alter energy storage homeostasis in fish. Increased energy stores were observed in native fish collected from Se-impacted field sites (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Goertzen et al., 2012). Dietary SeMet studies with adult zebrafish and rainbow trout also resulted in a greater accumulation of stored energy (Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013).

Chronic dietary Se exposure has been shown to alter swimming performance and energy homeostasis in adult fish as discussed above. However, little is known about the direct effects of dietary Se exposure in juvenile fish. Therefore, the objective of this study was to investigate the potential sublethal effects of subchronic dietary Se exposure on juvenile fish. To evaluate these effects, juvenile fathead minnow (*Pimephales promelas*) were fed diets spiked with Se in the form of SeMet for 60 days. Fathead minnow were selected for this experiment due to its use as a model organism in aquatic toxicity studies (Ankley and Villeneuve, 2006). Following the 60 day exposure period, fish were subjected to a swim performance challenge, from which the critical swim speed (U_{crit}), oxygen consumption (MO_2), and swim motion endpoints (tail beat frequency and amplitude) were determined. In addition to swimming performance, Se body burden, morphometrics (standard length, mass, condition factor), metabolic capacities (cost of transport, standard metabolic rate, active metabolic rate), indicators of energy storage capacity (whole body triglycerides and glycogen), and the physiological stress response (whole body cortisol) were determined.

2.2 Materials and methods

2.2.1 Test chemical

Seleno-L-methionine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Purity of the chemical was >98%.

2.2.2 Test species

20 days-post-hatch (dph) juvenile fathead minnow were randomly selected from an in-house stock in the Aquatic Toxicology Research Facility (ATRF) reared from eggs. Fish were housed in 9L aquaria contained in a diluter system to maintain a controlled

temperature ($23 \pm 1^{\circ}\text{C}$) and photoperiod (14h light and 10h dark). Fish were introduced randomly (30 fish per tank) into twelve 9L glass aquaria with continuous aeration and water flow rate (approximately one full water change per day). Aquaria were randomly assigned a treatment group with $n = 3$ replicate aquaria per treatment.

2.2.3 Diet preparation

Fish food (San Francisco Bay Brand Sally's BloodwormsTM, Newark, CA, USA) was spiked with different nominal concentrations of Se in the form of SeMet (3, 10, and 30 $\mu\text{g Se/g food dm}$). To prepare the experimental diets, freeze dried blood-worms were homogenized using a mortar and pestle. SeMet was weighed and dissolved in deionized water and added to the blood-worms. Diets were thoroughly mixed for 10 minutes and frozen at -80°C . The control diet was made by mixing the blood-worms with an equal volume of deionized water. Representative samples of each diet were selected for total Se analysis.

2.2.4 Experimental protocol

All experimental methods using live fish were carried out in the ATRF at the Toxicology Centre, University of Saskatchewan. All protocols in the present study were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Fish were fed twice daily at an estimated 5% body mass per day ration with either control or SeMet spiked food for 60 days. Fish were allowed to feed for 1 h, after which excess food was siphoned from the aquarium. All tanks were also siphoned to remove excess waste twice daily. Water samples ($n = 1$ for each aquarium) were collected 4 h after

feeding on day 30 for quantification of dissolved Se. Samples were filtered using 0.45 µm disposable filters, acidified to pH < 2 with ultrapure nitric acid, and stored in 8 ml Nalgene bottles at 4°C until Se analysis. Following the 60-day feeding trial, n = 5 fish from each treatment were euthanized using an overdose of MS-222 (1 g/l) and stored at -80°C for total Se analysis. Swimming performance, oxygen consumption, metabolic capacities, energy stores, and cortisol were determined in n = 12-24 fish per treatment, after which fish were euthanized and stored at -80°C. The remaining non-swam fish (not subjected to the U_{crit} challenge) were euthanized and stored at -80°C for subsequent analysis of whole body triglycerides, glycogen and cortisol. Prior to storage, standard body length, wet mass, and condition factor (wet mass/standard body length³ x 100) were determined for all fish.

2.2.5 Selenium analysis

Concentrations of total Se in control and SeMet spiked diets, and whole body fish tissue were determined by cold-digesting 100 mg aliquants of sample in Teflon vials with 5 ml of ultra-pure nitric acid and 1.5 ml of hydrogen peroxide. After digestion, samples were concentrated on a hot plate (<75°C), reconstituted in 5 ml of 2% ultra-pure nitric acid, and stored at 4°C until analysis. Whole body fish were lyophilized prior to cold digestion. Moisture content of whole body fathead minnow was $77.2 \pm 2.4\%$, determined by the difference in mass between fresh and lyophilized tissue. Total Se concentrations in food, fish tissue, and water were determined using inductively coupled plasma mass spectrometry (ICP-MS) at the Toxicology Centre. A method detection limit of 0.7 µg Se/g was determined from method blanks. Recovery of Se was determined using

certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada).

2.2.6 Swimming performance and oxygen consumption

Swim performance and oxygen consumption analysis was carried out using a modified Blazka-type, variable speed, model mini swim tunnel equipped with a DAQ-M automated oxygen measurement device (Loligo Systems, Tjele, Denmark). AutoRespTM 1 software (Loligo Systems) was used to calculate the rate of oxygen consumption (MO_2) during the swim challenge. The system consists of a 170 mL swim tunnel submerged in a 20 L buffer tank fed with aerated, dechlorinated municipal water from a 20 L temperature controlled water bath circulator (VWR International, Mississauga, ON, Canada). The temperature of the system was maintained at $23.5 \pm 0.1^\circ\text{C}$. MO_2 was measured using automated intermittent-flow respirometry in 10 minute loops. Each loop consisted of a 5 minute measuring phase, followed by a 4 minute flushing phase and a 1 minute waiting phase. Oxygen concentrations (O_2) were measured with a fibre optic dipping probe connected to a Fibox 3 minisensor oxygen meter (Precision Sensing GmbH, Regensburg, Germany) and MO_2 was subsequently calculated using the AutoRespTM 1 software. Steffensen et al. (1984) explains the MO_2 measuring principle in detail. Prior to the start of the swim trial, fish were placed in the swim tunnel and acclimated for 2 h at a low water velocity of 2.3 cm/s in order to establish a minimal rate of metabolism. During the swim challenge, fish were subjected to a step-wise incremental increase in swimming velocity of 7.7 cm/s every 20 minutes until exhaustion. Critical swimming speed (U_{crit}) was calculated using the method described by Brett (1964):

$$U_{crit} = V_p + ((t_f/t_i) \times V_i)$$

where V_p is the maximum velocity step the fish swam, V_i is the velocity increment (cm/sec), t_f is the elapsed time from the velocity increase to fatigue, and t_i is the time between velocity increase steps (Brett, 1964; Hammer, 1995). Fish cross-sectional area was less than 5% of the swim tunnel cross-sectional area, therefore the solid blocking effect correction was not applied. U_{crit} values were corrected for standard body length for each fish and were thus represented as body lengths per second (BL/s). Two MO_2 measurements were taken at each water velocity increment and averaged for statistical analysis. Following U_{crit} and MO_2 analysis, fish were euthanized using an overdose of MS-222, measured for mass and standard length, and stored at -80°C for subsequent analysis of whole body triglycerides, glycogen and cortisol.

2.2.7 Determination of COT, SMR, AMR, and F-AS

Cost of transport (J/kg/m) was calculated by multiplying MO_2 (mg O_2 /kg/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) (Videler, 1993). The standard metabolic rate (SMR) was calculated by extrapolating MO_2 values back to a water velocity of zero to approximate the SMR of a fish at rest. This was done by the use of a nonlinear, curve fitting regression analysis on a plot of swimming speed (m/s) versus MO_2 (Webber et al., 1998; Shingles et al., 2001; Thomas et al., 2013). The active metabolic rate is defined as the MO_2 at maximum sustainable swim speed during the swim challenge. Factorial aerobic scope (F-AS) was

calculated as AMR/SMR (Webber et al., 1998; Shingles et al., 2001; Killen et al., 2007; Thomas et al., 2013)

2.2.8 Swim motion analysis

Tail beat frequency and amplitude were measured concurrently during the swim performance tests with a Fastec Troubleshooter high-speed camera (Fastec Imaging, San Diego, CA, USA) mounted on a tripod above the swim tunnel. Tail beat frequency is a measure of the complete oscillation of the fish tail while tail beat amplitude measures the maximum tail stroke distance from the tip of the tail to the longitudinal body line. Both endpoints were determined at the highest completed velocity increment during the U_{crit} test. Videos were recorded at 250 frames per second when fish swam in a straight line. Adobe Premiere Element 2.0 (Adobe Systems, San Jose, CA, USA) was used to determine tail beat frequency. This software was also used to select three random frames of left and right tail beat amplitude for analysis with Image-Pro Express 6.0 (Media Cybernetics Inc., Bethesda, MD, USA). Values for left and right tail beat amplitude were added to obtain total amplitude values for each fish.

2.2.9 Tissue preparation

Whole body fathead minnow were taken from -80°C storage, thawed on ice and homogenized in 0.2M sodium citrate buffer with a Tissue Tearor (Fisher Scientific, Houston, TX, USA). Homogenate samples were stored at -80°C until triglyceride, glycogen and cortisol analyses. These colorimetric assays used a SpectraMAX 190 spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA) to read absorbances. Whole body homogenates were run in duplicate for each assay.

2.2.10 Quantification of triglycerides and glycogen

Concentrations of triglycerides in whole body juvenile fathead minnow were determined by the use of a commercially available kit prepared by Sigma-Aldrich (Oakville, ON, Canada). The assay followed the method of McGowan et al. (1983) and has been previously validated in our laboratory for measuring triglycerides in fish tissue samples (Weber et al., 2003; Kelly and Janz, 2008). A glycerol solution was used to generate the standard curve.

Glycogen concentrations in whole body juvenile fathead minnow were determined by the use of a modified method of Gómez-Lechón et al. (1996) validated for use in our laboratory (Weber et al., 2008; Goertzen et al., 2011; Massé et al., 2013; Thomas et al., 2013). Assay reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). Purified Type IX bovine liver glycogen was used to create the standard curve.

2.2.11 Cortisol assay

Cortisol concentrations were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA). Diethyl ether was used to extract cortisol from each whole body fish homogenate sample. Following extraction, a gentle stream of nitrogen gas was used to evaporate the ether and cortisol was reconstituted in phosphate buffer.

2.2.12 Statistical analyses

All data were analyzed using SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA). Data were assessed for normality using the Shapiro-Wilk test and homogeneity of variance was tested with Levene's test. Data that did not meet parametric assumptions were \log_{10}

transformed. Non-transformed data are shown in all figures. Data for U_{crit} , Se concentrations, fish morphometrics, swim motion, and metabolic capacities were tested by one-way ANOVA and Holm-Sidak post-hoc tests, when appropriate. Repeated measures ANOVA and Holm-Sidak post-hoc test was used to test for differences in MO_2 and COT of juvenile fish fed SeMet and control diets at each incremental water velocity of the U_{crit} test. Two-way ANOVAs were used to test the effect of Se treatment and swim status on whole body triglyceride, glycogen, and cortisol concentrations. When interactions between Se treatment and swim status were observed, one-way ANOVA was used to compare the effect of dietary Se concentration within each swim group, and also to compare the effect of the swim challenge within each exposure group. Holm-Sidak post-hoc test was used when appropriate. Data were expressed as mean \pm S.E.M. Differences were considered statistically significant at $p < 0.05$.

2.3 Results

2.3.1 Selenium analysis

Total Se concentrations in food, aquarium water, and whole body juvenile fathead minnow are shown in Table 2.1. The measured concentration of total Se in the non-spiked control diet was 2.8 $\mu\text{g Se/g dm}$. Total Se concentrations in the spiked food (nominal concentrations 3, 10, 30 $\mu\text{g Se/g dm}$) were 5.4, 9.9, and 26.5 $\mu\text{g Se/g dm}$ respectively, and were all significantly greater than the control diet ($p < 0.05$, Table 2.1). Concentrations of dissolved Se in aquarium water were below the ICP-MS detection limits for all tanks except the highest exposure group (26.5 $\mu\text{g Se/g dm}$) at 1.24 $\mu\text{g Se/L}$. Whole body Se concentrations in juvenile fathead minnow fed 2.8, 5.4, 9.9, and 26.5 $\mu\text{g Se/g dm}$ were 5.3, 6.6, 9.4, and 18.9 $\mu\text{g Se/g dm}$ respectively. Fish exposed in the two

highest treatment groups had significantly greater concentrations of whole body Se compared to control fish ($p < 0.05$, Table 2.1).

Table 2.1 Total selenium concentrations in food, whole body fathead minnow, and water. Fish were fed an estimated 5% body mass per day ration of control or SeMet spiked diet for 60 days. Data are mean \pm S.E.M. of $n = 3-5$.

Nominal diet ($\mu\text{g Se/g dm}$)	Measured Se in diet ($\mu\text{g Se/g dm}$)	Measured Se in whole body fish ($\mu\text{g Se/g dm}$)	Measured Se in water ($\mu\text{g Se/l}$)
1.0	2.83 ± 0.01	5.31 ± 0.33	<LOD
3.0	$5.35 \pm 0.04^*$	6.62 ± 0.76	<LOD
10.0	$9.91 \pm 0.33^*$	$9.42 \pm 0.75^{***}$	<LOD
30.0	$26.48 \pm 0.18^*$	$18.89 \pm 2.16^{***}$	1.24 ± 0.06

Significantly different from the control group using one-way ANOVA and Holm-Sidak post hoc test (*, $p < 0.05$; ***, $p < 0.001$).

2.3.2 Fish morphometrics

Standard length in juvenile fathead minnow exposed to control and SeMet spiked diets ranged from 19.2 mm to 20.1 mm (Table 2.2). The measured wet whole body mass of fish ranged from 149.1 mg to 168.5 mg (Table 2.2). There were no statistically significant differences in standard length and body mass among the control and treatment groups. The mean condition factors for each group fed SeMet spiked diets (1.97, 2.00 and 2.00) were significantly greater compared to the control group (1.89) ($p < 0.05$, $p < 0.01$ and $p < 0.01$, respectively; Table 2.2).

Table 2.2 Standard length, body mass and condition factor in juvenile fathead minnow fed different concentrations of selenomethionine for 60 days. Data are mean \pm S.E.M. of $n = 84-89$.

Measured Se in diet ($\mu\text{g Se/g dm}$)	Standard length (mm)	Wet mass (mg)	Condition factor
2.8	19.7 ± 0.2	149.5 ± 5.5	1.89 ± 0.02
5.4	20.1 ± 0.3	168.5 ± 6.9	$1.97 \pm 0.02^*$
9.9	19.2 ± 0.3	149.1 ± 6.0	$2.00 \pm 0.03^{**}$
26.5	19.7 ± 0.3	161.5 ± 7.1	$2.00 \pm 0.03^{**}$

Condition factor = (body mass/standard length³ x 100)

Significantly different from the control group using one-way ANOVA and Holm-Sidak post hoc test (*, $p < 0.05$; **, $p < 0.01$).

2.3.3 Swim performance and oxygen consumption

A significant decrease in swimming performance (U_{crit}) was detected in the 26.5 $\mu\text{g Se/g dm}$ exposure group when compared to the control group ($p < 0.001$, Fig. 2.1).

The U_{crit} value for fathead minnow fed 26.5 $\mu\text{g Se/g dm}$ was 3.29 BL/s, while the control group U_{crit} was 5.34 BL/s (Fig. 2.1). The U_{crit} value was also decreased in the 9.9 $\mu\text{g Se/g dm}$ treatment group (4.50 BL/s), however this result was not statistically significant compared to control fish.

Oxygen consumption (MO_2) was greater in fish fed SeMet spiked diets when compared to the control group (Fig. 2.2). At the first speed increment (0.02 m/s), the 26.5 $\mu\text{g Se/g dm}$ exposed group was significantly greater than the control group ($p < 0.05$, Fig. 2.2). At the remaining two speed increments (0.10 and 0.18 m/s), both the 9.9

and 26.5 $\mu\text{g Se/g dm}$ groups had significantly greater MO_2 compared to the control group ($p < 0.05$, Fig. 2.2).

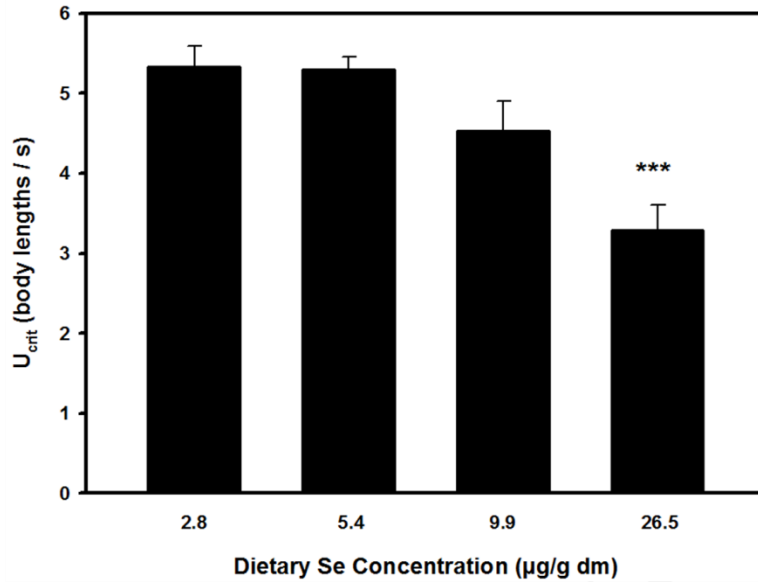


Figure 2.1 Critical swimming speed (U_{crit}) in juvenile fathead minnow fed different dietary concentrations of selenomethionine (2.8, 5.4, 9.9, and 26.5 $\mu\text{g Se/g}$ dry mass). Data are mean \pm S.E.M. of $n = 13$ -24 fish. ***Significantly different from control group using one-way ANOVA and Holm-Sidak post hoc test ($p < 0.001$).

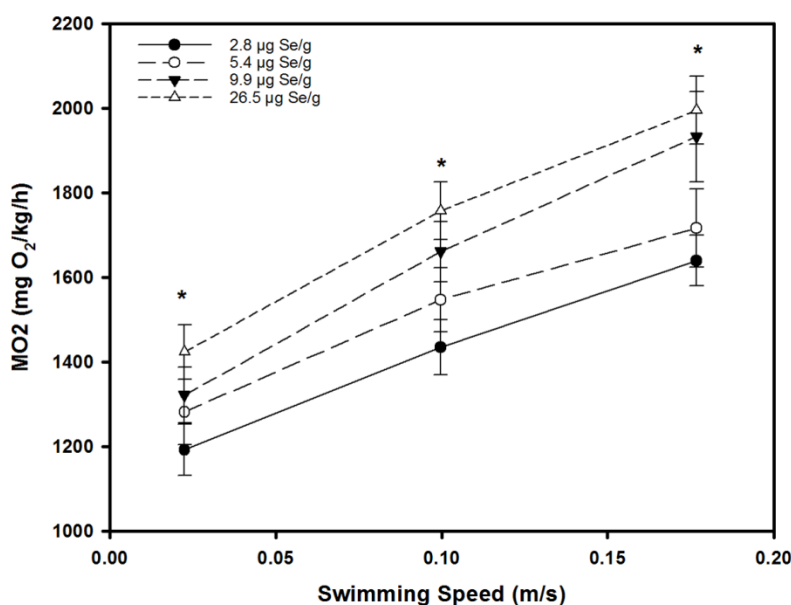


Figure 2.2 Oxygen consumption (MO_2) in juvenile fathead minnow fed different concentrations of selenomethionine. Data are mean \pm S.E.M. of $n = 13-24$ fish. *Significantly different from control group using one-way repeated measures ANOVA and Holm-Sidak post hoc test ($p < 0.05$).

2.3.4 Swim motion

Tail beat frequency and tail beat amplitude was measured at the highest completed swimming velocity increment. A significant decrease in tail beat frequency was observed in the 9.9 and 26.5 $\mu\text{g Se/g dm}$ fed fish compared to control fish ($p < 0.01$ and $p < 0.001$ respectively, Fig. 2.3A). Mean tail beat frequencies of control, 5.4, 9.9, and 26.5 $\mu\text{g Se/g dm}$ exposure groups were 14.7, 13.5, 11.7, and 10.8 tail beats/s respectively (Fig. 2.3A). Tail beat amplitude also demonstrated a significant decrease in the 9.9 and 26.5 $\mu\text{g Se/g dm}$ exposed fish compared to the control group ($p < 0.001$, Fig. 2.3B). Mean tail beat amplitude of control, 5.4, 9.9, and 26.5 $\mu\text{g Se/g dm}$ exposure groups were 8.1, 7.3, 5.8, and 5.2 mm respectively (Fig 2.3B).

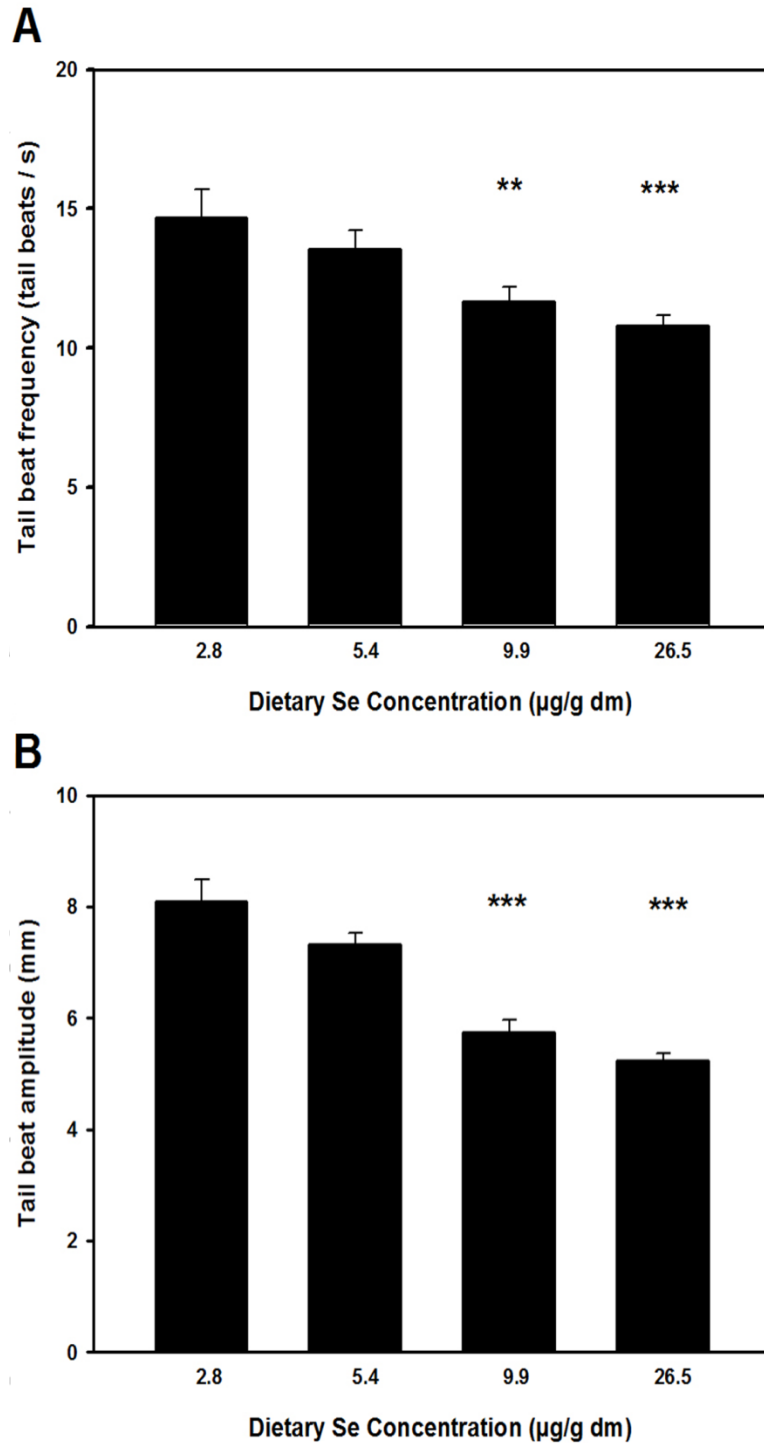


Figure 2.3 A, Tail beat frequency and B, tail beat amplitude (average stroke distance) in juvenile fathead minnow fed different concentrations of selenomethionine. Data are mean \pm S.E.M. of $n = 6-8$ fish. Significantly different from control group using one-way ANOVA and Holm-Sidak post hoc test (**, $p < 0.01$; ***, $p < 0.001$).

2.3.5 COT, SMR, AMR, and F-AS

Exposure to increasing concentrations of SeMet in the diet resulted in a consistent increase in the cost of transport (Fig. 2.4). Fish fed the spiked diet of 26.5 $\mu\text{g Se/g dm}$ had significantly increased COT compared to control fish at the first speed interval (0.02 m/s) ($p < 0.05$, Fig. 2.4). Both the 9.9 and 26.5 $\mu\text{g Se/g dm}$ exposure groups demonstrated a significantly increased COT compared to control fish at the remaining two speed increments ($p < 0.05$, Fig. 2.4).

Dietary SeMet exposure did not alter the SMR in control or treatment fish (Fig. 2.5). The mean MO_2 values for the SMR for the control, 5.4, 9.9, and 26.5 $\mu\text{g Se/g dm}$ treatment groups were 1152, 1257, 1270, and 1361 $\text{mg O}_2/\text{kg/h}$ respectively (Fig. 2.5). The AMR presented a similar trend of greater MO_2 along with increasing dietary Se concentration. AMR in the highest exposure group (1959 $\text{mg O}_2/\text{kg/h}$) was significantly greater than the control group (1701 $\text{mg O}_2/\text{kg/h}$) ($p < 0.01$, Fig. 2.5). There were no significant differences in the F-AS among exposure groups and controls.

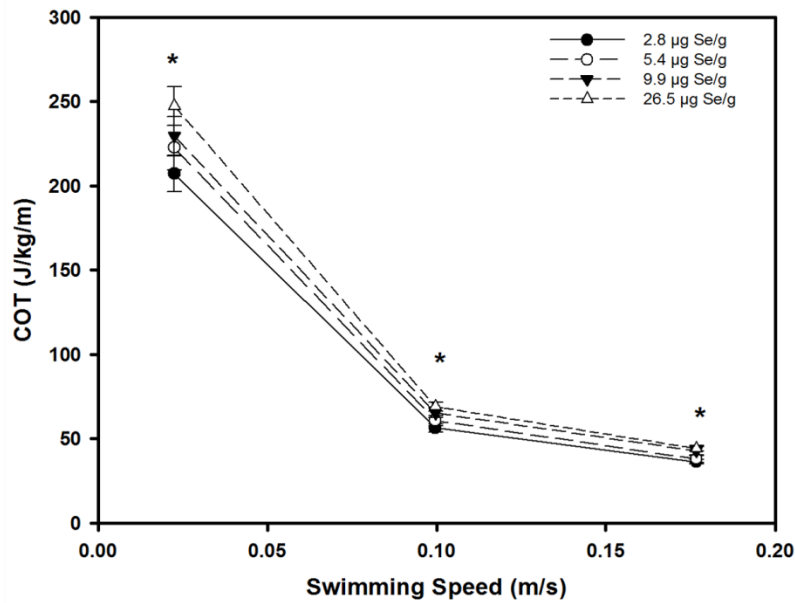


Figure 2.4 Cost of transport (COT) as a function of swimming speed in juvenile fathead minnow fed different concentrations of selenomethionine. Data are mean \pm S.E.M. of $n = 13-24$ fish. *Significantly different from control group using one-way repeated measures ANOVA and Holm-Sidak post hoc test ($p < 0.05$).

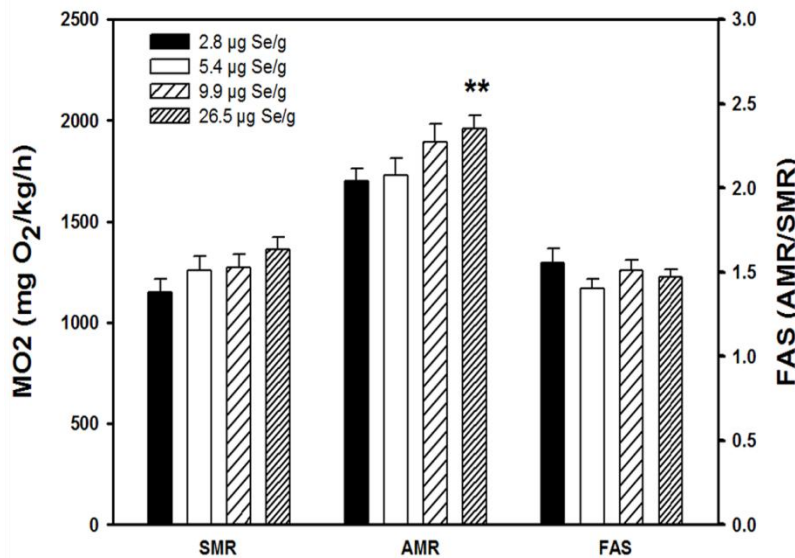


Figure 2.5 Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance MO_2 of unfed fish], active metabolic rate [AMR; the MO_2 at the maximum sustainable swimming speed in U_{crit}] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]) in juvenile fathead minnow fed control (2.8 $\mu\text{g Se/g dm}$) or Se spiked diets (5.4, 9.9, 26.5 $\mu\text{g Se/g dm}$) for 60 d. Data are mean \pm S.E.M. of $n = 13-24$ fish. **Significantly

different from control group using one-way ANOVA and Holm-Sidak post hoc test ($p < 0.01$).

2.3.6 Triglycerides and glycogen

Whole body concentrations of triglycerides were significantly greater in fish fed 5.4 $\mu\text{g Se/g dm}$ compared to control fish within the non-swam group not subjected to the swim performance test ($p < 0.001$, Fig. 2.6A). Two-way ANOVA detected a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.020$). There was no significant effect of dietary Se treatment on triglyceride concentration compared to controls in the swam group ($p = 0.132$). For the swim status factor, only the 5.4 $\mu\text{g Se/g dm}$ group showed a significant decrease in whole body triglyceride concentration in swam compared to non-swam fish ($p < 0.001$, Fig. 2.6A).

The concentration of whole body glycogen was significantly lesser in all treatment groups compared to controls in non-swam fish ($p < 0.05$, Fig. 2.6B). Two-way ANOVA detected a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.017$). There was no significant effect of dietary Se treatment on glycogen concentration in swam fish compared to controls ($p = 0.244$). Swim status had a significant effect on the 5.4 and 26.5 $\mu\text{g Se/g dm}$ groups, with an increased whole body glycogen concentration in swam fish compared to non-swam fish ($p < 0.001$ and $p < 0.05$ respectively, Fig. 2.6B).

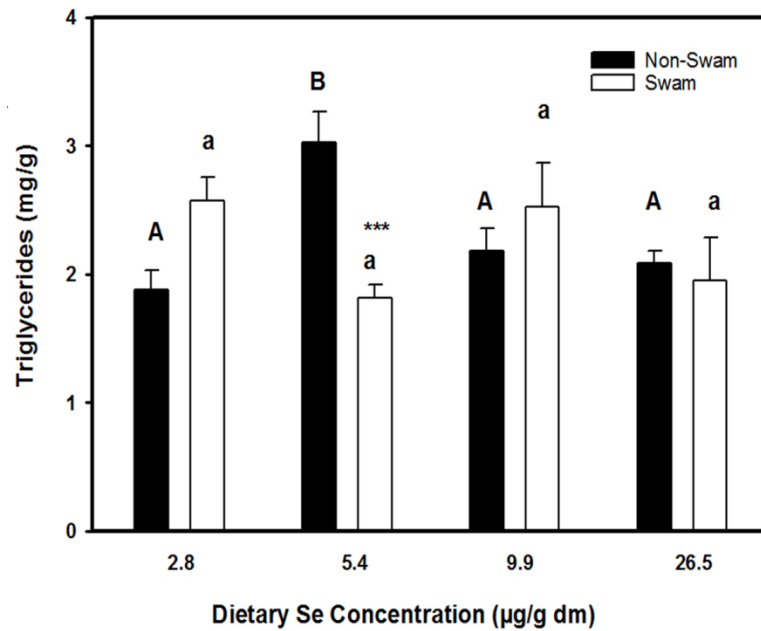
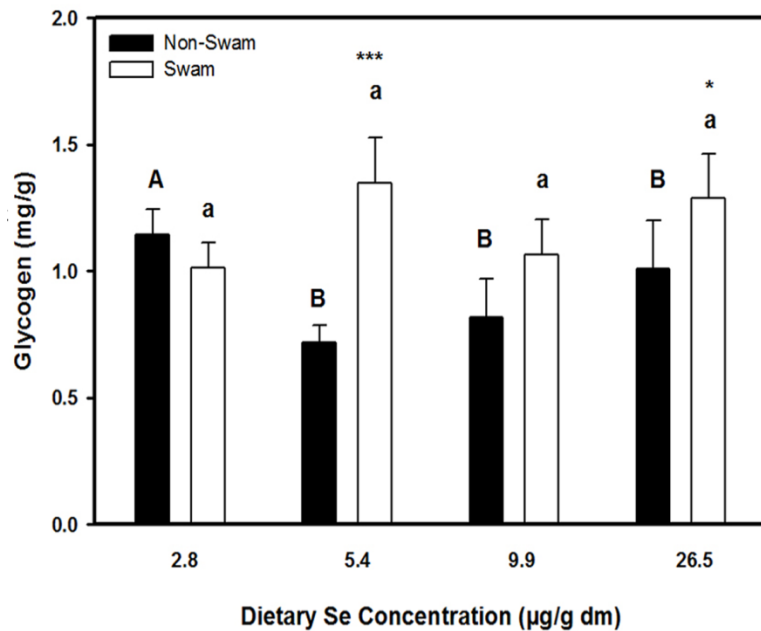
A**B**

Figure 2.6 A, Whole body triglycerides and B, whole body glycogen in juvenile fathead minnow fed control (2.8 µg Se/g dm) or selenomethionine spiked diets (5.4, 9.9, 26.5 µg Se/g dm) for 60 d. Bars represent the mean \pm S.E.M. of $n = 12-23$ fish. A, different uppercase letters denote a significant effect of dietary Se treatment on whole body triglyceride concentration in non-swam fish (one-way ANOVA with Holm-Sidak post hoc test, $p < 0.001$). Lowercase letters denote the absence of a significant effect of

dietary Se treatment on whole body triglyceride concentration in swam fish ($p = 0.132$). ***Significantly different from non-swam group using one-way ANOVA and Holm-Sidak post hoc test ($p < 0.001$). B, different uppercase letters denote significant effect of dietary Se treatment on whole body glycogen concentration in non-swam fish (one-way ANOVA with Holm-Sidak post hoc test, $p < 0.05$). Lowercase letters denote the absence of a significant effect of dietary Se treatment on whole body glycogen concentration in swam fish ($p = 0.217$). Significantly different from non-swam group using one-way ANOVA and Holm-Sidak post hoc test (*, $p < 0.05$; ***, $p < 0.001$).

2.3.7 Cortisol

There were no statistically significant differences in whole body cortisol concentrations among treatment groups in the non-swam group ($p = 0.246$, Fig. 2.7). In fish subjected to the swim performance test, the 5.4 $\mu\text{g Se/g dm}$ exposure group demonstrated a decreased whole body cortisol concentration compared to controls ($p < 0.01$, Fig. 2.7). Swim status had a significant effect on whole body cortisol, with the control, 9.9, and 26.5 $\mu\text{g Se/g dm}$ exhibiting increased whole body cortisol concentration in swam fish versus non-swam fish ($p < 0.01$, $p < 0.001$, and $p < 0.001$ respectively, Fig. 2.7).

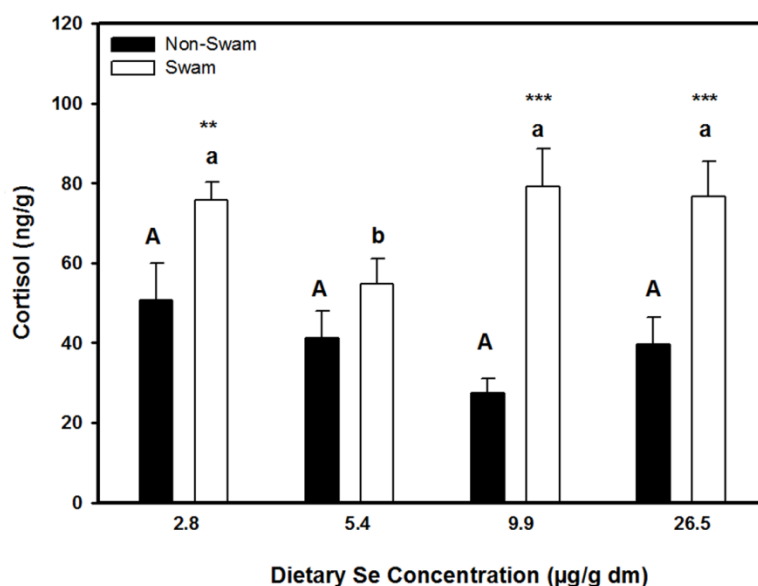


Figure 2.7 Whole body cortisol concentration in juvenile fathead minnow fed control (2.8 µg Se/g dm) or selenomethionine spiked diets (5.4, 9.9, 26.5 µg Se/g dm) for 60 d. Bars represent the mean \pm S.E.M. of $n = 12-23$ fish. Two-way ANOVA detected a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.049$).

Uppercase letters denote the absence of a significant effect of dietary Se treatment on whole body cortisol concentration in non-swam fish ($p = 0.246$). Different lowercase letters denote significant effect of dietary Se treatment on whole body cortisol concentration in swam fish (one-way ANOVA with Holm-Sidak post hoc test, $p < 0.01$).

** Represents a significant difference in whole body cortisol concentration between non-swam and swam fish in the control group (one-way ANOVA with Holm-Sidak post hoc test, $p < 0.01$). *** Represents a significant difference in whole body cortisol concentration between non-swam and swam fish in the 9.9 and 26.5 µg Se/g dm treatment groups (one-way ANOVA with Holm-Sidak post hoc test, $p < 0.001$).

2.4 Discussion

To our knowledge, the present study was the first to investigate swimming performance, aerobic metabolism, and energy homeostasis in juvenile fathead minnow in response to subchronic exposure to dietary Se. The results of this study suggest that both behavioural and physiological processes were affected. The most important findings were impaired swim performance along with increased oxygen consumption and potential impairment of aerobic metabolism, particularly in fish fed the highest SeMet

spiked diet (26.5 µg Se/g dm). The concentrations of Se in this study were environmentally relevant and were based on previous studies that demonstrated sublethal toxicities in adult zebrafish (Thomas and Janz, 2011; Thomas et al., 2013). Similar Se concentrations have been reported in fish and benthic invertebrates from Se impacted sites (Lemly, 1997b; Fan et al., 2002; Hamilton, 2004; Muscatello and Janz, 2009; Driedger et al., 2010). Selenium accumulation in whole body fish tissue occurred in a dose dependent manner and was proportional to concentrations of Se in the diet. Fish fed greater than 5.4 µg Se/g dm showed a significantly greater body burden of Se. Adult zebrafish exhibited a comparable increase in whole body Se concentrations in response to similar concentrations of dietary Se (Thomas and Janz, 2011; Thomas et al., 2013). Previous studies have also demonstrated accumulation of Se by cutthroat trout (*Oncorhynchus clarki*), Sacramento splittail (*Pogonichthys macrolepidotus*), and white sturgeon (*Acipenser transmontanus*) fed SeMet spiked diets (Teh et al., 2004; Tashjian et al., 2006; Hardy et al., 2010).

The dietary Se exposures in this study did not result in any significant differences in body mass or length of fish. However, condition factor was increased in each treatment group compared to controls. Similar results with regards to mass, length and condition factor were found in adult zebrafish exposed to comparable dietary SeMet concentrations (Thomas et al., 2013). In contrast to these results, another study in adult zebrafish reported that both body mass and length were increased while condition factor was unaffected in comparison to fish fed a control diet (Thomas and Janz, 2011). Rainbow trout administered a SeMet spiked diet demonstrated decreased body mass and fork length with greater condition factor, although a second study showed no effect on

fork length with increased body mass and condition factor (Wiseman et al., 2011a, 2011b). Juvenile Sacramento splittail exposed chronically to elevated dietary SeMet exhibited several deformities along with decreased mass, length and condition factor (Teh et al., 2004). In contrast, there were no deformities observed in fathead minnow in the present study.

Dietary SeMet exposure had a significant effect on swimming performance in juvenile fathead minnow. Fish fed greater than 9.9 $\mu\text{g Se/g dm}$ had significantly decreased U_{crit} compared to controls. A number of studies have examined swimming performance of adult and juvenile fish in response to aqueous selenite and selenate exposure, as well as dietary SeMet. Adult zebrafish exposed to aqueous selenite were reported to have reduced critical swim speed in repeat swimming experiments (Massé et al., 2013). Abnormal swimming behaviour such as lethargy and confinement to the tank bottom was reported in juvenile bluegill sunfish (*Lepomis macrochirus*) exposed to aqueous selenite and selenate mixtures (Cleveland et al., 1993). In the study by Cleveland et al. (1993), dietary SeMet exposure did not affect swimming behaviour. Sacramento splittail and white sturgeon exposed to elevated dietary SeMet experienced reduced swimming activity, swimming belly up and confinement to the tank bottom (Teh et al., 2004; Tashjian et al., 2006). In the study by Tashjian et al. (2006), juvenile white sturgeon experienced abnormal swimming behaviour at dietary Se concentrations of 41.7, 89.8, and 191.1 $\mu\text{g Se/g dm}$, much higher concentrations than were used for the present study (5.4 to 26.5 $\mu\text{g Se/g dm}$). Reasons for the differences in dietary Se concentrations resulting in decreased swim performance in the two experiments could be related to the methods used to investigate swimming behaviour. Tashjian et al. (2006) used an

observational approach to study normal swimming activity, while the present study used a forced swim test to investigate maximum swimming capacity. Adult zebrafish exposed to similar environmentally relevant concentrations of dietary Se had decreased swim performance when subjected to forced swim tests (Thomas and Janz, 2011; Thomas et al., 2013). Some studies have investigated repeat swim performance, in which fish are subjected to two successive U_{crit} tests with a short recovery period in between (Massé et al., 2013; Thomas et al., 2013). Repeat swim tests are used to investigate recovery rates of fish after exposure to contaminants and can provide a more sensitive measure of overall fish health than a single U_{crit} (Farrell et al., 1998; Jain et al., 1998). Future studies should consider the use of repeat swim performance experiments to further understand the effects of dietary SeMet exposure on U_{crit} in juvenile fishes.

A possible hypothesis for the reduction of critical swimming speed in juvenile fathead minnows in response to elevated dietary SeMet exposure could be impairment of muscle function. Impaired muscle function has been shown to negatively impact swim performance in fish (Beaumont et al., 2000; Shingles et al., 2001; McKenzie et al., 2003, 2009). For example, brown trout (*Salmo trutta*) exposed to sublethal concentrations of copper had reduced U_{crit} due to impairment of both red and white muscle function (Beaumont et al., 2000). Selenomethionine can arbitrarily substitute for methionine during protein synthesis (Schrauzer, 2000), potentially leading to altered muscle function. The shared characteristics of Se and sulphur allow Se to substitute for sulphur in methionine, resulting in altered tertiary structure and impaired function of proteins (Maier and Knight, 1994). Alternatively, oxidative stress induced by the catabolism of SeMet could also be responsible for muscle dysfunction. Selenomethionine is metabolised to

the reactive intermediate methylselenol (Wang et al., 2002; Palace et al., 2004; Spallholz et al., 2004), and methylselenol has been shown to cause oxidative stress in rainbow trout (Palace et al., 2004). Oxidative stress has been shown to impair the contraction of muscle fibres (Musarò et al., 2010), potentially causing muscle dysfunction responsible for decreased swim performance. In the present study, both tail beat frequency and tail beat amplitude significantly decreased in the two greatest exposure groups compared to controls. Because tail beat frequency and amplitude are functionally related to muscle contraction of the tail, the observed decrease further lends support to theory of muscle dysfunction being responsible for decreased U_{crit} . Fish with high tail beat amplitudes are likely to swim longer distances in a single beat of the tail than fish with low tail beat amplitudes (Bainbridge, 1958). This would suggest that control fish in the present study swam with a greater efficiency than the two highest Se exposed groups. Similarly, adult zebrafish exposed to a similar range of elevated dietary SeMet experienced a decrease in tail beat amplitude but not tail beat frequency (Thomas and Janz, 2011).

Another possible explanation for the decreased U_{crit} in the elevated Se exposed groups could be impaired aerobic metabolism. Alterations in MO_2 could indicate reduced aerobic capacity in fish (Watenpaugh and Beiting, 1985; Thomas et al., 2013). Indeed, respirometry is commonly used in tandem with swim tests to determine the energy requirements of fish exposed to environmental contaminants (De Boeck et al., 2006; Cheng and Farrell, 2007; Mager and Grosell, 2011; Marit and Weber, 2011; Massé et al., 2013; Thomas et al., 2013). In the present study, greater MO_2 values were observed in the 9.9 and 26.5 $\mu\text{g Se/g dm}$ treatment groups compared to controls. This increase in MO_2 is consistent with the decreased U_{crit} in the same treatment groups,

suggesting a possible link to impaired swim performance. Oxygen consumption has been reported to increase following exposures to both waterborne selenite and dietary SeMet (Lemly, 1993; Thomas et al., 2013). Juvenile bluegill sunfish exposed to both aqueous selenite and dietary SeMet had increased oxygen consumption compared to control groups (Lemly, 1993). Thomas et al. (2013) reported increased MO_2 in adult zebrafish in forced swim performance tests following exposure to environmentally relevant concentrations of dietary Se. Interestingly, adult zebrafish subjected to similar swim performance and respiration measurements after exposure to waterborne selenite did not exhibit a significant increase in MO_2 (Massé et al., 2013). This suggests that dietary rather than aqueous exposure may be a more sensitive exposure route with regards to forced U_{crit} and MO_2 .

Oxygen consumption is a physiologically relevant measurement that can be used to generate a number of other useful endpoints to assess the status of aerobic metabolism in fish. Cost of transport (COT) represents the energy required by a fish to move a unit of distance and is an index of overall swimming efficiency. Juvenile fathead minnow in the present study experienced an increase in cost of transport consistent with increased MO_2 and decreased U_{crit} , suggesting that fish in the highest two exposure groups were less efficient swimmers than control fish. The standard metabolic rate (SMR) is the oxygen consumption of fish at rest and represents the minimal metabolic rate for maintenance activity (Priede, 1985; Norin and Malte, 2011). Increased SMR can indicate greater energy requirements due to the biotransformation and excretion of toxicants and/or the repair of toxicant induced damage (Rowe et al., 2001). The active metabolic rate (AMR) is the oxygen consumption at maximum exercise and is the upper boundary

for aerobic metabolism (Norin and Malte, 2011). Aerobic scope (AS) and factorial aerobic scope (F-AS) are indicators of the absolute and proportional increases, respectively, in oxygen consumption rates in fish from rest to maximum exercise (Clark et al., 2013). F-AS was chosen for this study because it measures aerobic capacity in fish on a non-mass specific basis and also for its use in similar swim performance and respirometry experiments (Killen et al., 2007; Massé et al., 2013; Thomas et al., 2013). Adult zebrafish exposed to aqueous selenite experienced a decrease in AMR and AS in response to repeat swim performance tests (Massé et al., 2013), while dietary SeMet exposure to adult zebrafish resulted in increased COT with decreased AMR and F-AS during a U_{crit} challenge (Massé et al., 2013; Thomas et al., 2013). In the present study, juvenile fathead minnow demonstrated increased COT and increased AMR in the 26.5 μg Se/g dm exposure group. Interestingly, the trend in the adult zebrafish study by Thomas et al. (2013) was for increased SMR, with decreased AMR and F-AS, while we observed a trend of increased SMR and AMR, with no clear trend in F-AS. Both of these studies took place under similar exposure conditions and protocols, thus suggesting possible species and life stage sensitivity differences with respect to aerobic metabolism.

Energy storage capacity and the physiological stress response were investigated to further understand the impacts of dietary SeMet exposure on aerobic metabolism and energy usage. Greater accumulation of triglycerides and glycogen has been reported in fish exposed to SeMet spiked diets (Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013) and in fish collected from Se impacted sites (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Goertzen et al., 2012). Previous dietary SeMet studies in adult zebrafish showed dose dependent increases in both whole

body triglycerides and glycogen (Thomas and Janz, 2011; Thomas et al., 2013).

Interestingly, only the intermediate dose group, 5.4 $\mu\text{g Se/g dm}$, demonstrated the most overt significant alterations in energy storage capacity in the present study. The absence of any significantly greater accumulation of triglycerides and glycogen in the two highest exposure groups was unexpected and in contrast to previously reported U_{crit} and aerobic metabolism data (Thomas and Janz, 2011; Thomas et al., 2013). The decreased triglyceride concentrations in swam fish versus non-swam fish in the 5.4 $\mu\text{g Se/g dm}$ exposure group indicates that these fish are using triglycerides for their primary fuel source during swimming. Thomas et al. (2013) demonstrated the down regulation of β -hydroxyacyl coenzyme A dehydrogenase (HOAD) in adult zebrafish exposed to dietary SeMet. HOAD is a key energy metabolizing enzyme involved in β -oxidation of fatty acids. The potential impairment of HOAD could be responsible for the accumulation of triglycerides in the present study, however more information is required on the down regulation of HOAD in fathead minnows in response to dietary SeMet.

The decreased whole body glycogen concentrations in non-swam fish could be due to fish using glycogen as an energy source in response to SeMet exposure. This result is contrary to previous studies that have shown dose dependent increases in glycogen stores in response to dietary SeMet (Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013), and could represent a potential species and life stage sensitivity difference to dietary SeMet exposure in juvenile fathead minnow. Increased glycogen accumulation occurred in swam fish versus non-swam fish in the 5.4 and 26.5 $\mu\text{g Se/g dm}$ exposed groups. One possible reason for this is that these fish were using their triglyceride stores to fuel swimming, as anaerobic glycogen metabolism occurs only

after 80% U_{crit} (Hammer, 1995; Moyes and West, 1995). In addition, decreased cortisol concentrations in swam fish in the 5.4 $\mu\text{g Se/g dm}$ group could be responsible for the increased glycogen in the same group. Another possible hypothesis is the inhibition of citrate synthase (CS) activity. CS is commonly used as an indicator of tissue aerobic scope due to its role as a rate-limiting enzyme in the citric acid cycle. Goertzen et al. (2011) demonstrated decreased CS activity in juvenile fathead minnow exposed to Se-impacted site water. It is also possible that for both triglycerides and glycogen, whole body measures were not sensitive enough to detect tissue specific changes (e.g., muscle and liver) in energy homeostasis in this experiment.

Cortisol concentration was greater in swam fish versus non-swam fish, indicating that swim performance tests elicit the physiological stress response in fish exposed to elevated dietary SeMet. While not significant, there was a trend for decreased whole body cortisol among non-swam fish fed SeMet spiked diets. In contrast, zebrafish and rainbow trout demonstrated an increase in whole body and plasma cortisol concentrations, respectively, in response to elevated dietary SeMet exposure (Thomas and Janz, 2011; Wiseman et al., 2011b). As with triglycerides and glycogen, it is possible that whole body quantification of cortisol was not a sensitive enough measure to accurately determine the state of the physiological stress response, as cortisol is traditionally measured in plasma. While there appeared to be alterations in energy storage capacity and the physiological stress response in the present study, more information is needed to fully investigate the relationship between energy homeostasis, decreased U_{crit} , and altered aerobic metabolism in juvenile fathead minnow exposed to Se augmented diets.

In summary, dietary exposure to environmentally relevant concentrations of SeMet can reduce critical swimming speed and alter aerobic metabolism in juvenile fathead minnow. Altered aerobic metabolism and/or impaired muscle function may be responsible for the decrease in U_{crit} . In comparison to published literature, the dietary and whole body Se concentrations eliciting these responses in fathead minnow suggests that they are among the most sensitive endpoints in fish exposed directly to dietary and/or waterborne Se, and are not appreciably different from thresholds resulting in reproductive (i.e., larval deformities) impairment (Janz et al., 2010). Impaired swimming performance can potentially result in impacts to fitness and survivability due to increased predation risk, reduced food acquisition, and/or altered migration capacity of wild fish inhabiting Se contaminated aquatic ecosystems. These sublethal effects in juvenile fish may persist into adult life stages with a negative effect on recruitment and population sustainability.

CHAPTER 3

3.0 EFFECTS OF DIETARY SELENOMETHIONINE EXPOSURE ON SWIMMING PERFORMANCE, AEROBIC METABOLISM, AND ENERGY HOMEOSTASIS IN JUVENILE RAINBOW TROUT

This investigation was performed as the second of two studies in this thesis.

Similar to Chapter 2, it was designed to investigate the effects of a dietary Se exposure on juvenile fish. While Chapter 2 focused on the warm water fathead minnow species, the following chapter describes a dietary Se exposure on juvenile rainbow trout, a cold water species. Based on the results of the study described in Chapter 2, a different cold water species was selected for the second experiment to investigate any species sensitivity differences resulting from a subchronic dietary Se exposure. Larval rainbow trout were exposed to elevated concentrations of dietary Se in the form of SeMet for 37 days. Following exposure, samples were collected for Se analysis and fish were subjected to a swimming performance challenge to assess critical swim speed (U_{crit}), oxygen consumption (MO_2), cost of transport (COT), standard metabolic rate (SMR), active metabolic rate (AMR), and factorial aerobic scope (F-AS). Energy storage capacity was quantified via liver and muscle triglyceride and glycogen concentrations. The physiological stress response was measured by whole body cortisol concentrations. The observed results of this study suggest that exposure to environmentally relevant concentrations of elevated SeMet in the diet impairs swimming performance and alters energy homeostasis in juvenile rainbow trout. Impaired swimming performance may alter prey avoidance, food acquisition, migration and reproduction, and such alterations could affect the fitness and survival of wild fish.

3.1 Introduction

Selenium is an essential trace element with a narrow range between essentiality and toxicity. In fish, dietary levels of 0.1 to 0.5 $\mu\text{g Se/g dry mass (dm)}$ are sufficient for physiological homeostasis, however, concentrations greater than 3 $\mu\text{g Se/g dm}$ can result in increased Se accumulation and potential toxicity (Lemly, 1997b). Fish are especially of interest because they are among the most sensitive organisms to Se exposure (Janz et al., 2010; Janz, 2012). Selenium is present in the environment in soil deposits, sediments, black shale, coal, and phosphate rocks with natural processes such as volcanic activity, weathering of rocks, wildfires, and volatilization from plants and water bodies contributing to the redistribution of Se (Maher et al., 2010). Anthropogenic activities such as mining and milling operations, fossil fuel production, industrial manufacturing, and agriculture expansion have led to increased concentrations of Se in aquatic ecosystems (Janz et al., 2010; Maher et al., 2010). Selenium enters aquatic environment from point and non-point sources as inorganic selenite and selenate. These water soluble oxyanions are taken up by primary producers such as algae and biotransformed into organoselenide compounds such as selenocysteine and selenomethionine (SeMet) (Fan et al., 2002). Upon ingestion by consumer organisms, these organoselenides are incorporated into proteins and transferred to higher trophic level organisms following further ingestion. Therefore, the organic forms of Se have a much greater bioaccumulative potential than inorganic forms of Se. Selenomethionine is the dominant form of Se in the diet (Fan et al., 2002). Absorption and accumulation of SeMet occurs as a result of the shared molecular characteristics with the essential amino acid

methionine, for which SeMet substitutes during protein synthesis in a concentration dependent manner (Janz, 2012).

The maternal transfer of Se into eggs and subsequent yolk assimilation following ingestion of Se by adult fish is a key exposure pathway responsible for toxicity (Janz et al., 2010; DeForest and Adams, 2011). Larval and juvenile fish exhibit a number of developmental abnormalities when exposed to elevated Se concentrations (Lemly, 1997b; Muscatello et al., 2006; Muscatello and Janz, 2009). The utilization of SeMet and other organoselenides in developing fish embryos results in a characteristic suite of skeletal, craniofacial and fin malformations, along with edema, impaired growth and mortality (Lemly, 1998; Muscatello et al., 2006; Janz, 2012). As a result of this embryo and larval toxicity, the earliest life stages are also thought to be the most sensitive to direct dietary Se exposure (Muscatello et al., 2006; Tashjian et al., 2006). The dietary exposure pathway has been reported to affect a number of sublethal responses in juvenile and adult fish. Chronic dietary SeMet exposure has been shown to attenuate the physiological stress response, alter reproductive endocrine function, and significantly impair swim performance, aerobic metabolism, and energy homeostasis (Thomas and Janz, 2011; Wiseman et al., 2011a, 2011b; Thomas et al., 2013). Although previous studies have demonstrated toxic effects from chronic dietary Se exposure in adult fish, relatively little is known about the consequences of sublethal dietary Se exposure in juvenile fish.

Swimming performance and metabolic status are dynamic physiological traits in fish that can be used to generate a more biologically insightful evaluation of sublethal contaminant exposure than more traditional endpoints (Little and Finger, 1990; Kolok, 2001). Survival in the wild is dependent on swimming ability in regard to predator

avoidance, food acquisition, schooling, migration and mating and impairment of swimming performance could have consequences at the organismal, population, and ecosystem level, (Plaut, 2001; Scott and Sloman, 2004). Critical swimming speed (U_{crit}) is a commonly used incremental velocity test to determine maximum swim speed and aerobic capacity of fish (Brett, 1964; Webb, 1971; Farrell et al., 1998). Oxygen consumption (MO_2) is often determined in conjunction with U_{crit} testing via a swim tunnel respirator and provides a sensitive indicator of stress related to impaired aerobic metabolism (Watenpaugh and Beitingger, 1985; Kieffer et al., 1998). In addition, MO_2 can signal more subtle pathophysiological effects of contaminant exposure on the energetic cost of transport, standard and active metabolic rates, and aerobic scope as it relates to swimming performance (Videler, 1993; Hammer, 1995; Plaut, 2001; Shingles et al., 2001). Swim performance and respirometry thus provide sensitive and integrative indicators of sublethal Se exposure. Previous studies have demonstrated decreased U_{crit} and altered metabolic rates in response to waterborne selenite exposure and dietary SeMet exposure in adult zebrafish (*Danio rerio*) (Thomas and Janz, 2011; Thomas et al., 2013; Massé et al., 2013).

Swimming performance is directly connected to energy metabolism and homeostasis. The physiological stress response and energy homeostasis are highly interrelated processes. Cortisol is the major corticosteroid hormone in teleost fish and is essential to maintaining normal energy metabolism and homeostasis (Mommsen et al., 1999). Triglycerides and glycogen are the main forms of stored energy and are both used to fuel swimming (Tocher, 2003). Both plasma and whole body cortisol are useful measurements of physiological stress (Wendelaar Bonga, 1997; Ramsay et al., 2006).

Rainbow trout (*Oncorhynchus mykiss*) exposed chronically to SeMet in the diet demonstrated both an increase in basal plasma cortisol concentration and an attenuated cortisol release following an acute stressor, while adult zebrafish showed increased whole body cortisol concentrations following a similar dietary SeMet exposure (Thomas and Janz, 2011; Wiseman et al., 2011b). Cortisol targets the liver to mobilize triglyceride and glycogen stores under periods of stress. During U_{crit} testing, the aerobic catabolism of triglycerides act as the primary energy source for approximately 80% U_{crit} , after which glycogenolysis is initiated and the anaerobic catabolism of glycogen fuels the remainder of the swim challenge (Webb, 1971; Hammer, 1995; Moyes and West, 1995). Exposure to Se has been shown to alter energy storage homeostasis in fish. Increased energy stores were observed in native fish collected from Se-impacted field sites (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Goertzen et al., 2012). Adult zebrafish and rainbow trout have also demonstrated increased energy stores in response to environmentally relevant dietary SeMet exposures (Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013).

As outlined above, subchronic dietary SeMet exposure negatively impacts fish health via maternal transfer and direct dietary exposure in adult fish. Less is known about the relative sensitivity of fish based on diet-only SeMet exposure in juvenile fish. Therefore the objective of this study was to investigate the potential sublethal effects of a subchronic dietary Se exposure on a juvenile fish species. Juvenile rainbow trout were fed a diet spiked with Se in the form of SeMet for 37 days. Following the exposure period, fish were subjected to a swim performance challenge, from which the critical swim speed (U_{crit}), oxygen consumption (MO_2), and metabolic capacities (cost of

transport [COT], standard metabolic rate [SMR], active metabolic rate [AMR]) were determined. In addition to swimming performance, Se body burden, morphometrics (standard length, mass, condition factor), indicators of energy storage capacity (liver and muscle triglycerides and glycogen), and the physiological stress response (whole body cortisol) were determined.

3.2 Materials and methods

3.2.1 Test chemical

Seleno-L-methionine (purity >98%) was purchased from Sigma-Aldrich (Oakville, ON, Canada).

3.2.2 Test species

Female rainbow trout were reared from eggs obtained from a commercial supplier (Troutlodge, Sumner, WA, USA). Fourteen days-post-hatch juvenile fish were randomly selected for this experiment immediately following swim up and placed into four 719 L tanks that were divided with screened partitions into three sub-tanks of equal size. Each 719 L tank was supplied with continuous aeration and running water at a flow rate of 4 L/min and controlled temperature ($8 \pm 1^{\circ}\text{C}$) and photoperiod (14h light and 10h dark). Aquaria were randomly assigned a treatment group with $n = 3$ replicate aquaria per treatment.

3.2.3 Diet preparation

A commercial trout pellet (Martin Classic Sinking Fish Feed, Martin Mills Inc., Elmira, ON, Canada) was selected as the experimental diet. Fish feed was crushed and homogenized with a mortar and pestle and spiked with different nominal concentrations

of Se in the form of SeMet (3, 10, and 30 $\mu\text{g Se/g food dm}$ [38, 127, and 380 nmol/g]). SeMet was weighed and dissolved in nanopure water and added to the feed. Diets were thoroughly mixed for 10 minutes and frozen at -80°C . The control diet was made by mixing the trout pellets with an equal volume of nanopure water. Representative samples of each diet were selected for total Se analysis.

3.2.4 Experimental design

All experimental methods and protocols using live fish were carried out in the Aquatic Toxicology Research Facility at the Toxicology Centre, University of Saskatchewan and were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Fish were fed twice daily for a total ration of an estimated 5% body mass per day with either control or SeMet spiked food for 37 days. Fish were allowed to feed for 1 h, after which excess food and waste was siphoned from the aquarium. Water samples ($n=1$ for each aquaria) for dissolved Se determination were collected 4 h after feeding on day 20. Samples were filtered using $0.45\ \mu\text{m}$ disposable filters, acidified to $\text{pH} < 2$ with ultrapure nitric acid, and stored in 8ml Nalgene bottles at 4°C until Se analysis. Fish from each treatment group ($n = 5$) were randomly selected and euthanized using an overdose of MS-222 (1 g/l) upon completion of the feeding trial and stored at -80°C for total Se analysis. Swimming performance, oxygen consumption, bioenergetics, and metabolic capacities were measured in $n = 11-17$ fish per treatment. The remaining fish were euthanized and stored at -80°C for subsequent analysis of liver and muscle triglycerides and glycogen and whole body cortisol. Prior to storage, standard body

length, wet mass, and condition factor (wet mass/standard body length³ x 100) were determined for each fish.

3.2.5 Selenium analysis

Concentrations of Se in control and SeMet spiked diets, and whole body fish tissue were determined by cold-digesting 100 mg aliquants of sample in Teflon vials with 5 ml of ultra-pure nitric acid and 1.5 ml hydrogen peroxide. Prior to digestion, whole body fish tissue samples were lyophilized. Following digestion, samples were concentrated on a hot plate (<75°C), reconstituted in 5 ml of 2% ultra-pure nitric acid and stored at 4°C until analysis. Inductively coupled plasma mass spectrometry (ICP-MS) at the Toxicology Centre (University of Saskatchewan) was used to determine total Se concentrations in food, fish tissue, and water, as described previously (Thomas and Janz, 2011). An instrumental detection limit of 0.7 µg Se/g was determined from method blanks. Recovery of Se was determined using certified reference material (TORT-2, lobster hepatopancreas, NRC, Ottawa, ON, Canada).

3.2.6 Swimming performance and oxygen consumption

A modified Blazka-type, variable speed, model mini swim tunnel equipped with a DAQ-M automated oxygen measurement device (Loligo Systems, Tjele, Denmark) was used to measure swim performance and oxygen consumption in juvenile rainbow trout. The system consists of a 170 mL swim tunnel submerged in a 20 L buffer tank fed with aerated, dechlorinated municipal water from a 20 L temperature controlled water bath circulator (VWR International, Mississauga, ON, Canada). The temperature of the system was maintained at 8.0 ± 0.1°C. The MO₂ was measured using automated intermittent-flow respirometry in 10 minute loops with each loop consisting of a 5 minute

measuring phase, followed by a 4 minute flushing phase and a 1 minute waiting phase. A fibre optic dipping probe connected to a Fibox 3 minisensor oxygen meter (Precision Sensing GmbH, Regensburg, Germany) was used to measure concentrations of oxygen with AutoRespTM 1 software (Loligo Systems) calculating subsequent MO₂ concentrations. The MO₂ measurement principle is explained in detail by Steffensen et al. (1984). Fish were given an acclimation period of 2 h inside the swim tunnel at a minimal water velocity of 2.3 cm/s prior to the start of the swim trial in order to establish a minimal rate of metabolism. During the swim challenge, fish were subjected to a step-wise incremental increase in swimming velocity of 7.7 cm/s every 20 minutes until exhaustion. Critical swimming speed (U_{crit}) was calculated using the method described by (Brett, 1964):

$$U_{crit} = V_p + ((t_f/t_i) \times V_i)$$

where V_p is the maximum velocity step the fish swam, V_i is the velocity increment (cm/sec), t_f is the elapsed time from the velocity increase to fatigue, and t_i is the time between velocity increase steps (Brett, 1964; Hammer, 1995). Fish cross-sectional area was greater than 5% of the swim tunnel cross-sectional area therefore a correction for solid blocking effect was applied (Bell and Terhune, 1970). U_{crit} values were corrected for standard body length for each fish and thus represented as body lengths per second (BL/s). Two MO₂ measurements were taken at each water velocity increment and averaged for statistical analysis. Following U_{crit} and MO₂ analysis, fish were euthanized using an overdose of MS 222 (1 g/L), measured for mass, standard length and condition

factor, and stored at -80°C for subsequent analysis of liver and muscle triglycerides and glycogen.

3.2.7 Determination of COT, SMR, AMR, and F-AS

Cost of transport (J/kg/m) was calculated by multiplying MO_2 (mg O_2 /kg/s) by an oxycaloric value of 14.1 J/mg O_2 and then dividing by the corresponding swimming speed (m/s) to determine the COT for each fish at each swimming velocity (Videler, 1993). The standard metabolic rate (SMR) represents MO_2 values of a fish at rest. A nonlinear, curve fitting regression analysis on a plot of swimming speed (m/s) versus MO_2 was performed to extrapolate the MO_2 values back to a water velocity of zero in order to approximate the SMR of a fish at rest (Shingles et al., 2001; Thomas et al., 2013). The active metabolic rate (AMR) represents the maximum MO_2 during exercise and was calculated by averaging the MO_2 values at the highest sustainable swim speed during the swim challenge. Factorial aerobic scope (F-AS) is a measure of the maximum sustainable aerobic capacity of an organism and was calculated as AMR/SMR (Killen et al., 2007; Clark et al., 2013).

3.2.8 Tissue preparation

Whole body rainbow trout were taken from -80°C storage and thawed on ice. Liver and muscle tissue was dissected and immediately homogenized in 0.2M sodium citrate buffer (EMD Chemicals Inc., Gibbstown, NJ, USA) with a Tissue Tearor (Fisher Scientific, Houston, TX, USA). Homogenate samples were aliquoted and stored at -80°C until triglyceride and glycogen analysis. For whole body cortisol determination, rainbow trout were removed from -80°C storage, thawed on ice, homogenized in sodium citrate buffer and stored at -80°C until analysis. A SpectraMAX 190 spectrophotometer

(Molecular Devices Corp., Sunnyvale, CA, USA) was used to read the absorbances for these colorimetric assays. Tissue and whole body homogenates were run in duplicate for each assay.

3.2.9 Quantification of triglycerides and glycogen

Triglyceride (triacylglycerol) concentrations in liver and muscle of juvenile rainbow trout were determined by the use of a commercially available kit prepared by Sigma-Aldrich (Oakville, ON, Canada), following the method of McGowan et al. 1983. The assay has been previously validated in our laboratory for measuring triglycerides in whole body fish samples (Weber et al., 2003; Wiseman et al., 2011b; Goertzen et al., 2012; Massé et al., 2013). A glycerol solution was used to generate the standard curve.

Concentrations of liver and muscle glycogen in juvenile rainbow trout were determined using a modified method of Gómez-Lechón et al. (1996) validated for use in our laboratory (Thomas et al., 2013). Assay reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). Purified Type IX bovine liver glycogen was used to create the standard curve.

3.2.10 Cortisol assay

Cortisol concentrations were quantified with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Oxford Biomedical Research, Oxford, MI, USA). Diethyl ether was used to extract cortisol from each whole body fish homogenate sample. Following extraction, nitrogen gas was used to evaporate the ether and cortisol was reconstituted in phosphate buffer (Thomas and Janz, 2011).

3.2.11 Statistical analyses

All data were analyzed using SigmaStat 3.1 (SPSS Inc., Chicago, IL, USA). Normality and homogeneity of variance were tested by use of Shapiro-Wilk test and Levene's test respectively. Log_{10} transformations were performed on data that did not meet parametric assumptions. Data for U_{crit} , Se concentrations, fish morphometrics, swim motion, and metabolic capacities were tested by one-way ANOVA and Holm-Sidak post-hoc tests, when appropriate. Two-way ANOVAs were used to test the effect of Se treatment and swim status on liver and muscle triglyceride and glycogen concentrations. There were no interactions between dietary factor and swim status factor for all two-way ANOVAs. Holm-Sidak post-hoc test was used when appropriate where significant differences were detected by two-way ANOVA to compare SeMet exposed groups versus control fish and non-swam versus swam fish. Data were expressed as mean \pm S.E.M. Differences were considered statistically significant at $p < 0.05$.

3.3 Results

3.3.1 Selenium analysis

Total Se concentrations in food, aquarium water, and whole body juvenile rainbow trout are shown in Table 3.1. The measured concentration of total Se in non-spiked control diet was 1.0 $\mu\text{g Se/g dm}$ (13 nmol/g). Total Se concentrations in the SeMet spiked food (nominal concentrations 3, 10, 30 $\mu\text{g Se/g dm}$) were 4.1, 11.2, and 26.1 $\mu\text{g Se/g dm}$ respectively and were all significantly greater than the control diet ($p < 0.001$, Table 3.1). Concentrations of dissolved Se in aquarium water were 0.3 $\mu\text{g/L}$ for all treatment groups (Table 3.1). Whole body Se concentrations in juvenile rainbow trout fed 1.0, 4.1, 11.2, and 26.1 $\mu\text{g Se/g dm}$ were 1.6, 3.8, 9.7, and 19.9 $\mu\text{g Se/g dm}$

respectively, and all SeMet exposed groups were significantly greater than control fish ($p < 0.001$, Table 3.1).

Table 3.1 Total selenium concentrations in food ($\mu\text{g/g}$ dry mass), water ($\mu\text{g/L}$), and whole body rainbow trout ($\mu\text{g/g}$ dry mass). Fish were fed an estimated 5% body mass per day ration of control or SeMet spiked diets for 37 days. Data are mean \pm S.E.M. of $n = 3-5$.

Nominal diet ($\mu\text{g Se/g dm}$)	Measured Se in diet ($\mu\text{g Se/g dm}$)	Measured Se in whole body fish ($\mu\text{g Se/g dm}$)	Measured Se in water ($\mu\text{g Se/L}$)
1.0	1.0 ± 0.21	1.6 ± 0.07	0.3 ± 0.01
3.0	$4.1 \pm 0.08^{***}$	$3.8 \pm 0.16^{***}$	0.3 ± 0.01
10.0	$11.2 \pm 0.64^{***}$	$9.7 \pm 1.06^{***}$	0.3 ± 0.05
30.0	$26.1 \pm 1.55^{***}$	$19.8 \pm 2.19^{***}$	0.3 ± 0.01

Significantly different from the control group using one-way ANOVA and Holm-Sidak post hoc test ($^{***}, p < 0.001$).

3.3.2 Fish morphometrics

Standard length in juvenile rainbow trout exposed to control and SeMet spiked diets ranged from 4.5 to 4.7 cm (Table 3.2). The measured wet whole body mass of fish ranged from 1.5 to 1.7 g (Table 3.2). The mean condition factor for control and SeMet exposed fish ranged from 1.56 to 1.62 (Table 3.2). There were no statistically significant differences in any of the morphometric measures among exposed and control fish.

Table 3.2 Standard length, body mass and condition factor in juvenile rainbow trout fed different concentrations of selenomethionine for 37 days. Data are mean \pm S.E.M. of n = 86-89.

Measured Se in diet ($\mu\text{g Se/g dm}$)	Standard length (cm)	Wet mass (g)	Condition factor
1.0	4.50 ± 0.05	1.50 ± 0.05	1.59 ± 0.02
4.1	4.66 ± 0.05	1.65 ± 0.06	1.56 ± 0.01
11.2	4.68 ± 0.06	1.67 ± 0.06	1.57 ± 0.02
26.1	4.66 ± 0.05	1.70 ± 0.06	1.62 ± 0.04

Condition factor = (body mass/standard length³ x 100)

3.3.3 Swim performance and oxygen consumption

Swimming performance (U_{crit}) was significantly reduced in the 11.2 and 26.1 $\mu\text{g Se/g dm}$ exposure groups compared to controls ($p < 0.01$, Fig. 3.1). Rainbow trout fed 11.2 and 26.1 $\mu\text{g Se/g dm}$ had U_{crit} values of 3.8 BL/s and 3.6 BL/s, respectively, while the control group U_{crit} was 4.7 BL/s (Fig. 3.1).

The relationship between MO_2 and swimming speed is shown in Figure 3.2. There were no clear trends in MO_2 with dietary SeMet exposure and increasing swim speed, and no significant differences in MO_2 were detected among control and SeMet exposed fish (Fig. 3.2).

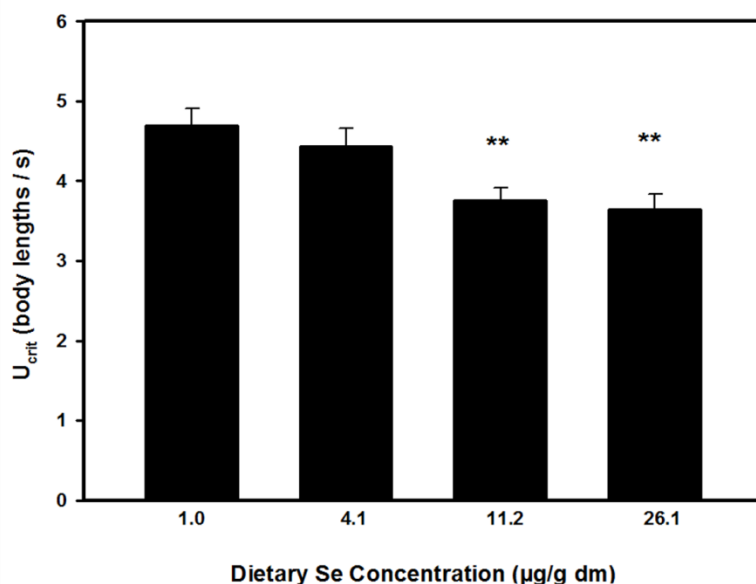


Figure 3.1 Critical swimming speed (U_{crit}) in juvenile rainbow trout fed different concentrations of selenomethionine (1.0, 4.1, 9.9, and 26.1 $\mu\text{g Se/g}$ dry mass) for 37 days. Data are mean \pm S.E.M. of $n = 13$ -16 fish. **Significantly different from control group using one-way ANOVA and Holm-Sidak post hoc test ($p < 0.01$).

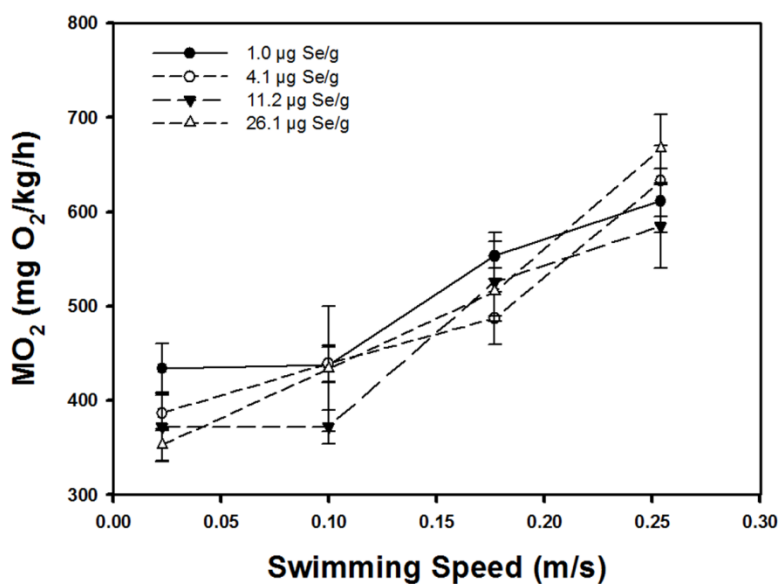


Figure 3.2 Oxygen consumption (MO_2) in juvenile rainbow trout fed different concentrations of selenomethionine. Data are mean \pm S.E.M. of $n = 11$ -15 fish.

3.3.4 COT, SMR, AMR, and F-AS

Cost of transport decreased with increasing swimming velocity. However, there were no statistically significant differences in COT between SeMet exposed groups and controls (Fig. 3.3). The SMR in rainbow trout exposed to increasing concentrations of SeMet in the diet appeared to be lower than control fish. However, there were significant differences among exposure groups (Fig. 3.4). The mean MO_2 values for the SMR for the control, 4.1, 11.2, and 26.1 $\mu\text{g Se/g dm}$ treatment groups were 404, 361, 327, and 338 $\text{mg O}_2/\text{kg/h}$ respectively (Fig. 3.4). Dietary SeMet exposure did not alter the AMR when comparing control and fish fed the SeMet spiked diets (Fig. 3.4). The mean MO_2 values for the AMR for the control, 4.1, 11.2, and 26.1 $\mu\text{g Se/g dm}$ treatment groups were 613, 602, 604, and 594 $\text{mg O}_2/\text{kg/h}$ respectively (Fig. 3.4). The aerobic scope presented a trend of increasing F-AS with increasing dietary Se concentration (Fig. 3.4). However, there were no statistically significant differences in F-AS among control and SeMet fed groups.

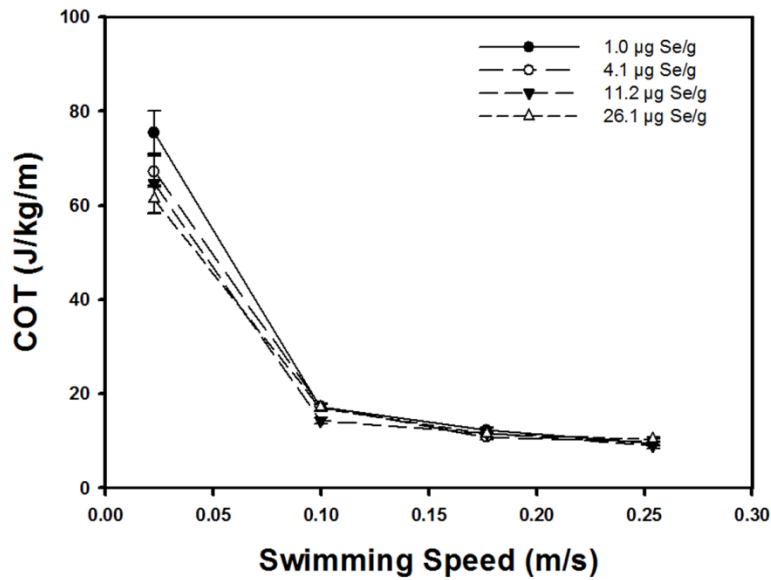


Figure 3.3 Cost of transport (COT) as a function of swimming speed in rainbow trout fed different concentrations of selenomethionine. Data are mean \pm S.E.M. of $n = 11-15$ fish.

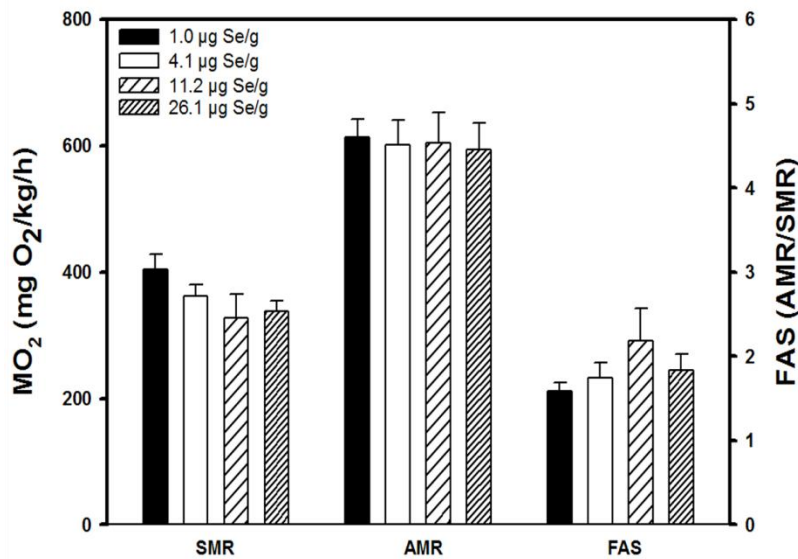


Figure 3.4 Metabolic capacities (standard metabolic rate [SMR; the minimal maintenance MO_2 of unfed fish], active metabolic rate [AMR; the MO_2 at the maximum sustainable swimming speed in U_{crit}] and factorial aerobic scope [F-AS; the ratio of AMR to SMR]) in juvenile rainbow trout fed control ($1.0 \mu\text{g Se/g dm}$) or Se spiked diets (4.1 , 11.2 , $26.1 \mu\text{g Se/g dm}$) for 37 d. Data are mean \pm S.E.M. of $n = 11-15$ fish.

3.3.5 Bioenergetics

Concentrations of triglycerides in the liver were significantly altered by both the dietary SeMet treatment factor and the swim status factor ($p < 0.001$ for Se treatment factor and $p < 0.001$ for swim status factor in two-way ANOVA; Fig. 3.5A). Two-way ANOVA did not detect a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.777$). In non-swam fish, liver triglyceride concentrations were significantly lower in all SeMet exposed groups compared to controls ($p < 0.05$, Fig. 3.5A). In swam fish, a trend of decreasing liver triglyceride concentration was apparent, however only the 11.2 $\mu\text{g Se/g dm}$ exposure group was statistically significant compared to controls ($p < 0.05$, Fig. 3.5A). Concentrations of liver triglycerides were lower in swam fish versus non-swam fish for all treatment groups with only the control and 11.2 $\mu\text{g Se/g}$ treatment groups displaying statistical significance ($p < 0.05$, Fig. 3.5A).

Concentrations of triglycerides in muscle were significantly altered by both dietary SeMet treatment factor and the swim status factor ($p < 0.001$ for Se treatment factor and $p = 0.028$ for swim status factor in two-way ANOVA; Fig. 3.5B). Two-way ANOVA did not detect a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.633$). Although two-way ANOVA showed a significant effect of dietary treatment factor on concentrations of muscle triglycerides in rainbow trout, post hoc tests were unable to demonstrate any statistically significant differences between dietary SeMet treatment groups compared to controls in both non-swam and swam fish. For the swim status factor, muscle triglyceride concentrations were significantly decreased in swam versus non-swam fish in all treatment groups ($p < 0.05$, $p < 0.01$, and $p < 0.001$, Fig. 3.5B).

Liver glycogen concentrations in juvenile rainbow trout exposed to dietary SeMet were significantly altered by the swim status factor but not the dietary treatment factor ($p < 0.001$ for swim status factor and $p = 0.334$ for dietary treatment factor in two-way ANOVA; Fig. 3.6A). Two-way ANOVA did not detect a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.903$). Concentrations of glycogen in liver was greater in swam fish versus non-swam fish in all treatment groups, however only the 4.1 $\mu\text{g Se/g dm}$ exposure group demonstrated a statistically significant difference ($p < 0.05$, Fig. 3.6A).

Concentrations of glycogen in muscle were significantly altered by both the dietary SeMet treatment factor and the swim status factor ($p < 0.001$ for Se treatment factor and $p = 0.017$ for swim status factor in two-way ANOVA; Fig. 3.6B). Two-way ANOVA did not detect a significant interaction between dietary Se treatment factor and swim status factor ($p = 0.925$). In non-swam fish, muscle glycogen concentrations were significantly decreased in the 4.1 and 11.2 $\mu\text{g Se/g}$ exposed groups compared to controls ($p < 0.05$, Fig. 3.6B). Among fish subjected to the swim challenge, there were no statistically significant differences in muscle glycogen concentrations in SeMet exposed fish compared to controls (Fig. 3.6B). For the swim status factor, muscle glycogen concentrations were significantly greater in swam versus non-swam fish in all treatment groups ($p < 0.01$, and $p < 0.001$, Fig. 3.6B).

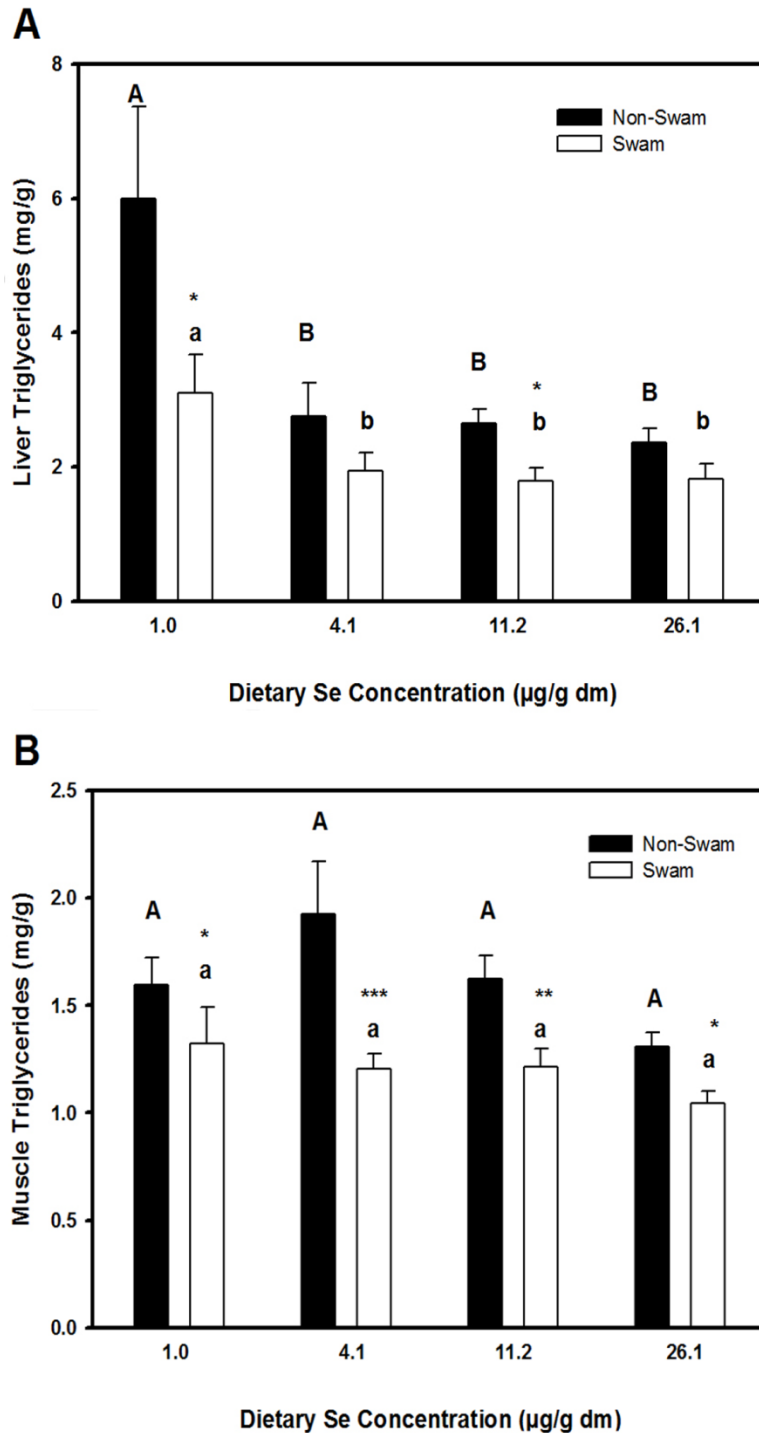


Figure 3.5 A, Liver triglycerides and B, muscle triglycerides in juvenile rainbow trout fed control (1.0 µg Se/g dm) or selenomethionine spiked diets (4.1, 11.2, 26.1 µg Se/g dm) for 37 d. Bars represent the mean \pm S.E.M. of $n = 8-17$ fish. A, different uppercase letters denote a significant effect of dietary Se treatment on liver triglyceride concentration in non-swam fish (two-way ANOVA with Holm-Sidak post hoc test, $p < 0.01$). Different lowercase letters denote a significant effect of dietary Se treatment on

liver triglyceride concentration in swam fish ($p < 0.05$). *, Significantly different from non-swam group using two-way ANOVA and Holm-Sidak post hoc test ($p < 0.05$). B, significantly different from non-swam group using two-way ANOVA and Holm-Sidak post hoc test (*, $p < 0.05$; **, $p < 0.01$ ***, $p < 0.001$).

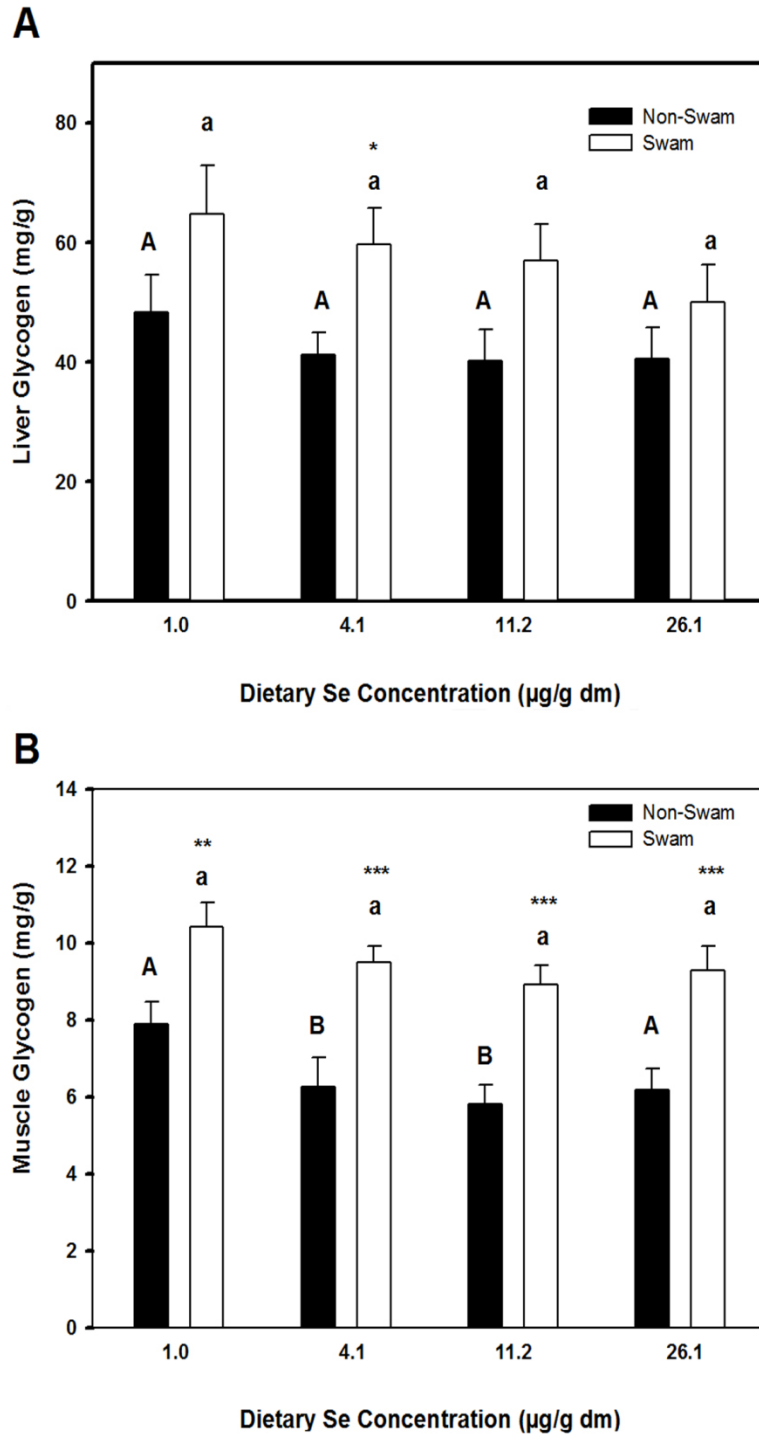


Figure 3.6 A, Liver glycogen and B, muscle glycogen in juvenile rainbow trout fed control (1.0 $\mu\text{g Se/g dm}$) or selenomethionine spiked diets (4.1, 11.2, 26.1 $\mu\text{g Se/g dm}$) for 37 d. Bars represent the mean \pm S.E.M. of $n = 12-16$ fish. A, *significantly different from non-swam group using two-way ANOVA and Holm-Sidak post hoc test ($p < 0.05$). B, different uppercase letters denote a significant effect of dietary Se treatment on liver glycogen concentration in non-swam fish (two-way ANOVA with Holm-Sidak post hoc test, $p < 0.05$). Significantly different from non-swam group using two-way ANOVA and Holm-Sidak post hoc test (**, $p < 0.01$; ***, $p < 0.001$).

3.3.6 Cortisol

Whole body cortisol concentrations of rainbow trout exposed to dietary SeMet are shown in Fig. 3.7. Cortisol concentrations appeared lower in all treatment groups compared to controls, however, one-way ANOVA did not reveal any statistically significant difference among treatments.

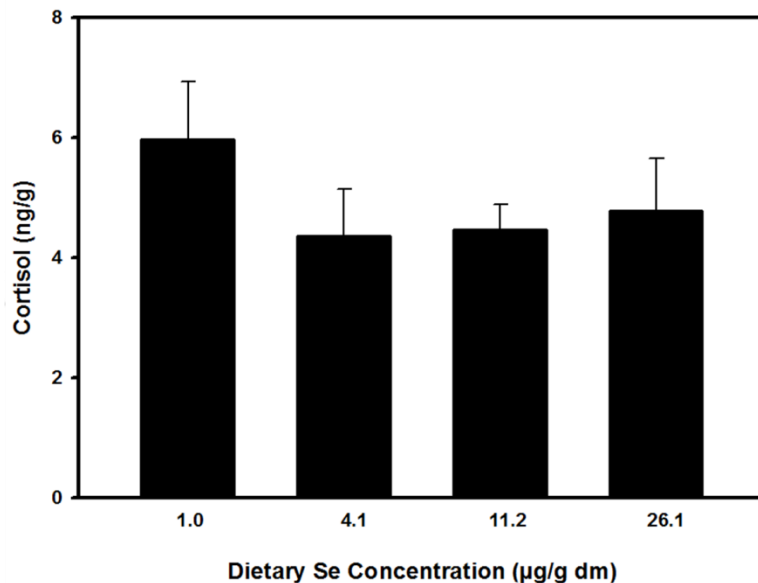


Figure 3.7 Whole body cortisol in juvenile rainbow trout fed different concentrations of selenomethionine (1.0, 4.1, 9.9, and 26.1 $\mu\text{g Se/g dry mass}$) for 37 days. Data are mean \pm S.E.M. of $n = 10-13$ fish.

3.4 Discussion

The major findings of the present study were a decrease in critical swimming speed (U_{crit}) and altered energy homeostasis in juvenile rainbow trout following subchronic dietary SeMet exposure. The results suggest that both behavioural and physiological processes were affected. Dietary Se concentrations used in this study were environmentally relevant and were modelled after previous studies conducted in adult zebrafish that were shown to elicit sublethal effects (Thomas and Janz, 2011; Thomas et al., 2013). Whole body Se accumulation was proportional to dietary Se concentrations and increased in a dose-dependent manner. Fish fed greater than 1.0 $\mu\text{g Se/g dm}$ had significantly greater tissue concentrations of Se. Previous studies have reported accumulation of Se in muscle tissue of crucian carp (*Carassius auratus gibelio*) and whole body of Sacramento splittail (*Pogonichthys macrolepidotus*), juvenile green (*Acipenser medirostris*) and white sturgeon (*Acipenser transmontanus*) and cutthroat trout (*Oncorhynchus clarki*) subjected to dietary SeMet exposure (Teh et al., 2004; Tashjian et al., 2006; Zhou et al., 2009; Hardy et al., 2010; De Riu et al., 2014). Adult zebrafish exposed to comparable treatments of dietary SeMet also demonstrated a similar concentration-dependent increase in whole body Se (Thomas and Janz, 2011; Thomas et al., 2013).

There was no significant effect of dietary SeMet exposure on the body mass, standard length, and condition factor of juvenile rainbow trout in the present study. Larval rainbow trout exposed to SeMet spiked diets for 90 days showed a decrease in both body mass and fork length (Vidal et al., 2005). Other dietary SeMet studies conducted in rainbow trout have demonstrated decreased body mass and fork length with

greater condition factor, along with no effect on fork length and increased body mass and condition factor (Wiseman et al., 2011a, 2011b). Adult zebrafish exposed to SeMet spiked diets resulted in increased condition factor with no effect on body mass and length, while a second study showed increased body mass and length with no change in condition factor (Thomas and Janz, 2011; Thomas et al., 2013). The varying results with regards to fish morphometrics in response to dietary SeMet exposure in this experiment and others in the literature suggest that these measures alone are perhaps not the most reliable indicators of sublethal dietary SeMet toxicity.

To our knowledge, this was the first study investigate forced swimming performance, aerobic metabolism, and energy homeostasis in juvenile rainbow trout in response to a sublethal exposure to dietary Se. Swimming performance in juvenile rainbow trout was decreased in response to dietary SeMet exposure. In comparison to published literature, the dietary and whole body Se concentrations responsible for this response in rainbow trout indicate that it is a sensitive endpoint in fish exposed directly to dietary Se, and is not considerably different from thresholds resulting in larval deformities (Janz et al., 2010). Adult zebrafish experienced similar decreases in U_{crit} in both repeat swim and single U_{crit} tests in response to dietary SeMet exposure (Thomas and Janz, 2011; Thomas et al., 2013). Juvenile white sturgeon and Sacramento splittail exposed to increased dietary SeMet concentrations demonstrated impaired swimming ability in the form of confinement to the tank bottom, reduced activity and swimming belly up (Teh et al., 2004; Tashjian et al., 2006). The studies conducted by Teh et al. (2004) and Tashjian et al. (2006) reported abnormal swimming behaviour at much higher dietary SeMet concentrations than the present study. The methods used to measure

swimming performance in these experiments could help explain the differences observed. Both Teh et al. (2004) and Tashjian et al. (2006) used an observational approach to study normal swimming behaviour while the present study used a forced swim test to investigate maximum swimming speed and aerobic metabolism. A number of studies have also examined the effects of waterborne selenite and selenate on swimming performance in fish. Decreased U_{crit} was reported in adult zebrafish subjected to repeat swim performance testing following exposure to waterborne selenite (Massé et al., 2013). Cleveland et al. (1993) reported impaired swimming behaviour in juvenile blue gill sunfish (*Lepomis macrochirus*) exposed to aqueous mixtures of selenite and selenate, however, an exposure to dietary SeMet in the same study was unable to elicit any negative effects on swimming behaviour.

The present study was unable to explain the mechanism responsible for decreased U_{crit} , however, potential hypotheses can be proposed for the observed impaired swim performance. Selenomethionine can substitute for methionine in a random dose-dependent manner during protein synthesis (Stadtman, 1974; Schrauzer, 2000; Janz, 2012). As a result, disulfide linkages are disrupted and tertiary structure of proteins may be altered, potentially resulting in dysfunction of muscle proteins (Maier and Knight, 1994; Lemly, 1998). This altered amino acid substitution could lead to impaired muscle contraction resulting in reduced U_{crit} . Adult zebrafish exposed to SeMet spiked diets had decreased tail-beat amplitude compared to unexposed fish (Thomas and Janz, 2011). Thomas and Janz (2011) proposed that because tail-beat amplitude is functionally related to muscle contraction of the tail, decreased tail-beat amplitude in fish fed SeMet indirectly supports the hypothesis that impaired muscle function is responsible for

decreased U_{crit} . Furthermore, the catabolism of SeMet could result in muscle dysfunction due to oxidative stress. Methylselenol, a reactive intermediate, has been shown to cause oxidative stress in rainbow trout (Wang et al., 2002; Palace et al., 2004; Spallholz et al., 2004). Musarò et al. (2010) demonstrated that oxidative stress impaired muscle contraction. Taken together with the improper substitution of SeMet for methionine, it is possible that impaired muscle function was responsible for decreased swim performance.

Swim respirometry is a commonly used method to assess aerobic metabolism of fish during swim performance testing. Alterations in MO_2 could signify reduced aerobic capacity in fish (Watenpaugh and Beiting, 1985). Adult zebrafish exposed to dietary SeMet experienced an increase in MO_2 along with decreased U_{crit} (Thomas et al., 2013). In addition, juvenile bluegill sunfish had increased oxygen consumption resulting from both aqueous selenite and dietary SeMet exposure (Lemly, 1993). These results give credence to the hypothesis that decreased swimming performance could be due to impaired aerobic metabolism. Interestingly in the present study, there was no discernible trend in MO_2 values for juvenile rainbow trout. Oxygen consumption did increase with swimming speed, however there were no statistically significant differences in exposed groups versus controls at each speed increment. A possible reason for this could be that fishes swimming in flowing water will often prefer to take advantage of any differences in flow to reduce energetic costs associated with swimming movements (Liao, 2007). Cold-acclimated rainbow trout have been shown to be unwilling or unable to swim continuously for extended periods (Kieffer et al., 1998), potentially impacting MO_2 measurements in swim challenge experiments. Oxygen consumption was also unaffected by increasing swim speed in juvenile Pacific bluefin (*Thunnus orientalis*) at 8°C (Blank et

al., 2007). In addition, large individual variability has been reported in fish MO_2 values in previous respirometry studies (Marras et al., 2010; Taguchi and Liao, 2011; Thomas and Janz, 2011). Another explanation could be due to species and life stage sensitivity differences in swimming behaviour of juvenile rainbow trout that while reflected in decreased U_{crit} , was not a result of alterations in MO_2 . Similarly to the MO_2 results in the present study, there was no significant effect of dietary SeMet exposure on COT. Cost of transport is a measure of the overall swimming efficiency. There was a slight trend of decreased COT in SeMet exposed groups compared to controls, however this trend was not consistent at all speeds of the swim performance test. These results suggest that dietary SeMet exposure did not significantly impact the swimming efficiency of juvenile rainbow trout. The SMR is the oxygen consumption of fish at rest and represents the minimal metabolic rate required to maintain normal activity while the AMR is the oxygen consumption at maximum exercise and is the upper boundary for aerobic metabolism (Priede, 1985; Norin and Malte, 2011). The AMR was not significantly altered in this study. The factorial-aerobic scope (F-AS) is a non-mass specific measure of aerobic capacity in fish from rest to maximum exercise (Killen et al., 2007; Clark et al., 2013). While not statistically significant, there was an increasing trend in F-AS in juvenile rainbow trout exposed to dietary SeMet in the present study. An important factor to consider is the water temperature at which fish were exposed. Rainbow trout are a cold water species and were exposed at 8°C in the present study. Similar studies involving dietary SeMet exposure in adult zebrafish, a warm water fish species, were conducted at 28°C and reported impaired swimming performance along with increased MO_2 , COT, SMR (Thomas et al., 2013). These results are contrary to our observations in

juvenile rainbow trout and could be influenced by exposure water temperature. For example, there is much more dissolved oxygen at 8°C vs 28 °C, and therefore oxygen may not have been a limiting factor when it came to swimming performance and aerobic metabolism for the juvenile rainbow trout exposed to dietary SeMet in the present study. All together, these results suggest that in this case, altered/impaired aerobic metabolism may not have been responsible for impaired swimming performance. Further studies regarding the relationship between aerobic metabolism and dietary SeMet exposure in juvenile rainbow trout are required to accurately determine their influence on swim performance testing.

Previous studies in adult zebrafish have demonstrated altered bioenergetic status in response to dietary SeMet exposure (Thomas and Janz, 2011; Thomas et al., 2013). In addition, fish collected from Se impacted sites had increased stores of triglycerides and glycogen (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010; Goertzen et al., 2012). In the present study, concentrations of triglycerides in liver were significantly decreased in SeMet exposed rainbow trout versus controls in non-swam and swam fish. This result suggests that rainbow trout could be mobilizing triglyceride stores in the liver to combat the stress from the dietary SeMet exposure. Liver triglyceride concentrations were also decreased in swam fish versus non-swam fish in all groups, with the controls and 11.2 µg Se/g dm groups displaying statistical significance, suggesting that fish were mobilizing liver triglycerides to fuel swimming. There were no discernible trends or significant differences in muscle triglyceride concentrations among SeMet exposed fish versus controls in either non-swam or swam fish. However, muscle triglycerides were decreased in all treatment groups following the swim challenge,

suggesting that intramuscular aerobic catabolism of triglycerides was used to fuel swimming as described previously (Moyes and West, 1995; Webb, 1971).

Concentrations of glycogen in the liver of rainbow trout exposed to dietary SeMet were unchanged in both non-swam and swam treatment groups compared to controls. When it came to the swim status factor, liver glycogen was increased in all swam fish with the only significant difference occurring in the 4.1 $\mu\text{g Se/g dm}$ exposure group. In muscle tissue, glycogen concentrations were decreased in exposed non-swam fish with no change in swam fish. Similar to liver glycogen concentrations, muscle glycogen was increased in swam fish versus non-swam fish in all treatment groups. Elevated glycogen in swam fish could be the result of triglycerides acting as the primary fuel source for swimming as anaerobic glycolysis is only activated after 80% U_{crit} (Hammer, 1995; Moyes and West, 1995). Decreased muscle glycogen in non-swam exposed rainbow trout could be due to fish using glycogen stores to cope with the increased energy demands resulting from dietary SeMet exposure.

Interestingly, adult zebrafish exposed to environmentally relevant concentrations of SeMet in the diet showed a dose-dependent increase in both whole body triglycerides and glycogen (Thomas and Janz, 2011; Thomas et al., 2013). Immature rainbow trout exposed to dietary SeMet also demonstrated increased muscle triglycerides and glycogen (Wiseman et al., 2011b). In addition, spottail shiner (*Notropis hudsonius*) collected from Se-impacted sites had increased liver triglycerides (Goertzen et al., 2012). Based on these studies, we expected to see similar results in juvenile rainbow trout exposed to dietary SeMet. However, the results in the present study run in contrast to the previously published literature (Bennett and Janz, 2007; Kelly and Janz, 2008; Driedger et al., 2010;

Thomas and Janz, 2011; Wiseman et al., 2011b; Goertzen et al., 2012; Thomas et al., 2013). This outcome suggests possible species and life stage sensitivity differences in juvenile rainbow trout with respect to energy homeostasis following dietary SeMet exposure.

While not statistically significant, there was a trend of decreasing whole body cortisol among SeMet exposed fish. Interestingly, immature rainbow trout exposed for 126 days to elevated dietary SeMet showed an increase in basal plasma cortisol, and an attenuated cortisol response following an acute stressor. These results suggest a potential life stage sensitivity difference in rainbow trout exposed to dietary SeMet. Adult zebrafish exposed to SeMet augmented diets showed an increase in whole body cortisol concentration, further reinforcing the possibility of species and life-stage sensitivity differences (Thomas and Janz, 2011). It could be possible that in the present study, whole body measurements of cortisol may not have been sensitive enough to accurately determine the effects on the physiological stress response as cortisol is typically measured in plasma. Also, Pacific cod (*Gadus macrocephalus*) acclimated at 4°C had decreased concentrations of plasma cortisol compared to fish acclimated at 11°C (Hanna et al., 2008), suggesting that temperature may be a factor in determining the physiological stress response and energetic cost in fish. Indeed, Clarke and Johnston (1999) have reported that fish inhabiting cold water systems have an energetic advantage due to decreased energetic maintenance costs. Further research is required to determine any influence of exposure temperature when it comes to the effects of dietary SeMet exposure on the energy storage capacity and physiological stress response in juvenile fish.

3.5 Conclusion

Exposure to environmentally relevant concentrations of SeMet in the diet significantly decreased juvenile rainbow trout swim performance. Critical swim speed proved to be a sensitive method of identifying sublethal effects of dietary SeMet exposure in juvenile rainbow trout. Impaired muscle function may be the mechanism responsible for decreased U_{crit} . Reduced swimming ability may eventually impact fish sustainability in Se-impacted ecosystems by impeding essential behaviours such as predator avoidance, migration, reproduction, and food acquisition. The effects of dietary SeMet exposure on aerobic capacity are unclear and require further investigation. Altered energy homeostasis may indicate differences in species and life stage sensitivity to dietary SeMet exposure.

CHAPTER 4

4.0 GENERAL DISCUSSION

4.1 Project rationale and summary

Recent studies have reported fish exposed to elevated concentrations of SeMet in the diet have altered swimming performance, aerobic metabolism, physiological stress response and bioenergetic status (Teh et al., 2004; Tashjian et al., 2006; Thomas and Janz, 2011; Wiseman et al., 2011b; Thomas et al., 2013). While the toxic effects of elevated dietary SeMet on adult fish have been demonstrated, less is known about the sublethal effects of dietary SeMet exposure on juvenile fish species. Due to the well-characterized toxic effects of Se on larval fish, the earliest life stages are thought to be the most sensitive to dietary SeMet exposure (Muscatello et al., 2006; Muscatello and Janz, 2009; Tashjian et al., 2006). Therefore, laboratory studies focusing on diet-only exposures on different species of juvenile fish warranted further investigation. The purpose of this thesis was to further characterize the effects of subchronic dietary SeMet exposure in two juvenile fish species, fathead minnow and rainbow trout.

Overall, the major observation of this study was that dietary SeMet exposure caused a dose-dependent decrease in swimming performance in both juvenile fathead minnow and rainbow trout. In addition, dietary SeMet exposure resulted in altered bioenergetic status in both species, with significant effects on stored energy in tissues resulting from both dietary treatment and swimming status. However, effects on aerobic metabolism and oxygen consumption varied considerably between the two species. Juvenile fathead minnow experienced significant alterations in the endpoints assessing

aerobic metabolic homeostasis, while rainbow trout did not display any significant trends, despite reduced swimming capacity and altered bioenergetic status.

4.2 Comparison between juvenile fathead minnow and juvenile rainbow trout dietary SeMet exposures

4.2.1 Selenium concentrations

In both experiments, the measured concentrations of Se in the diets were appreciably close to the intended nominal concentrations of 1.0, 3.0, 10.0, and 30.0 $\mu\text{g Se/g dm}$. Commercially available blood-worms and trout pellets were used as the experimental diets for fathead minnow and rainbow trout, respectively. Previous studies investigating dietary SeMet exposure on adult zebrafish have used commercial flake food as the experimental diet (Thomas and Janz, 2011; Thomas et al., 2013). In natural habitats, one food source of fathead minnows includes chironomid species. Therefore, chironomid larvae (blood-worms) were chosen to represent an environmentally relevant food source as well as being feasible for use the laboratory. The measured Se concentration in blood-worm control diet (basal 1.0 $\mu\text{g Se/g dm}$) was 2.8 $\mu\text{g Se/g dm}$. This was unexpected and is higher than the desired nominal concentration. The spiking of blood-worms with SeMet to attain the targeted nominal concentrations proved difficult. Previous blood-worm SeMet spiking trials yielded control groups with elevated Se (data not shown). It took multiple attempts with different brands of blood-worms until the desired nominal concentration was approximated. In the end, San Francisco Bay Brand Sally's BloodwormsTM yielded a concentration of 2.8 $\mu\text{g Se/g dm}$ and were selected as the control diet for the fathead minnow study. While 2.8 $\mu\text{g Se/g dm}$ approaches toxicity thresholds for fish ($>3.0 \mu\text{g Se/g dm}$, Lemly, 1997b), it can still be

considered a valid control in the present thesis. Teh et al. (2004) reported that juvenile Sacramento splittail exposed to 2.7 and 6.6 $\mu\text{g Se/g dm}$ did not significantly differ in mass or length compared to a control group of 0.4 $\mu\text{g Se/g dm}$. Larval rainbow trout exposed to 4.6 $\mu\text{g Se/g dm}$ for 60 days did not affect mass or fork length compared to controls fed a diet of 0.23 $\mu\text{g Se/g dm}$ (Vidal et al., 2005). In addition, mass was not significantly different in cutthroat trout exposed to 3.2 $\mu\text{g Se/g dm}$ compared to a 1.2 $\mu\text{g Se/g dm}$ control group. Therefore, it is reasonable to assume that 2.8 $\mu\text{g Se/g dm}$ is a valid control and would not act as another treatment group in the fathead minnow experiment. A potential reason for the elevated control concentration could include other species of Se present in the food and thus contributing to a higher overall Se concentration. The ICP-MS method of determining Se concentration in the spiked feed determined total Se only, therefore it is possible that species of Se other than SeMet were present in the food before spiking, contributing to a higher total Se than expected. Selenomethionine is the dominant form of Se found in the diet of fish, however there are a number of classes of Se present in aquatic biota, such as elemental Se, selenate/selenite, selenides/selenols and selenoxides (Fan et al., 2002; Rigby et al., 2014). X-ray absorption spectroscopy (XAS)/x-ray absorption near-edge spectroscopy (XANES) is often used to determine the speciation of Se in aquatic biota (Franz et al., 2011; Misra et al., 2010, 2012; Phibbs et al., 2011a, 2011b). Unfortunately, the use of XAS/XANES was out of the scope of this project. While a number of species of Se may exist in fish tissue and their prey in natural habitats, the primary aim of the present research was to investigate the direct effects of dietary SeMet on fish fitness. Future studies involving dietary SeMet exposure and its effects on fish fitness could use XAS/XANES to look at

Se speciation in the diet in more detail. Other potential food sources and methods of Se spiking could be used to ensure accurate delivery of SeMet to the experimental diet, such as live cultures of chironomids raised on a diet of selenized yeast (Teh et al., 2004).

Concentrations of Se in aquaria ranged from below detection limit to 1.3 $\mu\text{g Se/l}$, suggesting that Se was taken up into fish tissues and not excreted in any great amounts. These results were consistent with other dietary SeMet exposure studies (Thomas and Janz, 2011; Thomas et al., 2013), and indicates that fish received the dose of Se from the diet and were not at risk of any inadvertent aqueous Se exposure.

Whole body tissue concentrations of Se increased with increasing dietary Se concentration and in fathead minnow ranged from 5.3 to 18.9 $\mu\text{g Se/g dm}$, and in rainbow trout from 1.6 to 19.8 $\mu\text{g Se/g dm}$. These results are consistent with Se tissue concentrations reported in previous dietary SeMet exposures in fish (Thomas and Janz, 2011; Thomas et al., 2013; Wiseman et al., 2011b). In mature fish, the measurement of egg Se concentrations is the best indication of toxic hazard, however the sampling time is critical as reproductive physiology is dynamic. Concentrations greater than 15 $\mu\text{g Se/g dm}$ in eggs warrant concern for further investigation (Janz et al., 2010). The EC10 values for egg Se concentration range from between 15 – 25 $\mu\text{g Se/g dm}$ in a number of species with cutthroat trout being the most sensitive and fathead minnow the most tolerant species (Janz et al., 2010). The whole body Se concentrations in juvenile fathead minnow and rainbow trout in the present study were sufficient enough to elicit impaired swim performance and aerobic metabolism, and altered energy homeostasis. Therefore, the results of this study suggest that these are sensitive sublethal endpoints in fish

exposed to SeMet in the diet, and are similar to previously reported Se thresholds in eggs that were responsible for reproductive toxicity (Janz et al., 2010).

4.2.2 Growth and condition

Dietary SeMet exposure did not have a marked effect on indices of growth and condition in the two studies in this thesis. Wet mass and standard length were unaffected in both juvenile fathead minnow and rainbow trout exposed to SeMet in the diet.

Increased condition factor was observed in fathead minnow, however condition factor in rainbow trout was unchanged. The published literature on dietary SeMet exposures in fish reveals a great deal of variability in the growth response and overall condition. Adult zebrafish exposed to similar concentrations of dietary SeMet for 90 days showed increased body mass and length with no change in condition factor in one experiment, while another exposure demonstrated no changes in body mass and length with increased condition factor (Thomas and Janz, 2011; Thomas et al., 2013). Vidal et al. (2005) reported increased mass and fork length in larval rainbow trout following a 90 day dietary SeMet exposure. In addition, two studies investigating dietary SeMet exposure on rainbow trout showed increased body mass and condition factor, and decreased body mass, length, and increased condition factor following a 126 day exposure (Wiseman et al., 2011a, 2011b). Decreased body mass, length, and condition factor have been observed in juvenile Sacramento splittail and juvenile white sturgeon exposed to SeMet in the diet for 5 to 9 months and 4 to 8 weeks respectively (Teh et al., 2004; Tashjian et al., 2006). Other studies have reported no change in condition factor in juvenile bluegill in response to a 90 day dietary SeMet exposure, as well as increased body mass of crucian carp exposed to dietary SeMet for 30 days (Cleveland et al., 1993; Zhou et al.,

2009). Therefore, morphometric data alone is not a sensitive and reliable indicator of dietary SeMet exposure in fish. Growth and condition of fish may be highly dependent on exposure conditions and experimental protocols such as the feeding regime, water temperature, exposure length, life stage and fish species. While morphometric endpoints are useful in determining the overall health of fish, they are not robust enough on their own to provide a consistent and reliable indication of the sublethal effects of dietary SeMet exposure.

4.2.3 Swimming performance and aerobic metabolism

Critical swim speed tests have been shown to be an environmentally relevant and sensitive measure of contaminant exposure in a number of fish species (Beaumont et al., 1995; Hopkins et al., 2003; McKenzie et al., 2007; Mager and Grosell, 2011; Marit and Weber, 2011). Field and laboratory studies have demonstrated the utility of U_{crit} testing to assess the aerobic fitness of fish in response to a variety of conditions (Goertzen et al., 2011, 2012; Massé et al., 2013; Thomas et al., 2013). The present study was designed to investigate the effects of a subchronic dietary SeMet exposure on the swimming performance and metabolic capacities of two juvenile fish species in a laboratory setting. Both species experienced decreased U_{crit} in response to elevated dietary SeMet exposure. Fathead minnow had decreased U_{crit} at the highest dose, while rainbow trout had decreased U_{crit} at the two highest treatment groups.

The intended exposure period for rainbow trout was 60 days however, due to the growth rates, fish size and swim tunnel size, the exposure period was limited to 37 days. Interestingly, this dietary exposure period was still sufficient enough to elicit decreased swim performance in rainbow trout subjected to the two highest treatments compared to

controls. Fathead minnow also demonstrated increased MO_2 , COT, and AMR in dietary SeMet exposed groups while rainbow trout were unaffected by dietary SeMet exposure in the endpoints used to assess aerobic metabolism during swimming performance. One possible reason for the observed decrease in swimming performance and alterations to aerobic metabolism in juvenile fathead minnow and juvenile rainbow trout could be attributed to exposure temperature. The fathead minnow exposure was conducted at $23 \pm 1^\circ\text{C}$ and the rainbow trout were exposed at $8 \pm 1^\circ\text{C}$. Temperature is inversely related to dissolved oxygen, therefore there is much greater dissolved oxygen at 8°C than at 23°C . Perhaps the decreased availability of oxygen at 23°C was a limiting factor to juvenile fathead minnow that led to altered aerobic metabolism and impaired swim performance. Along the same lines, it follows that due to the abundance of dissolved oxygen at 8°C , the rate of oxygen consumption may not have been a limiting factor when it came to swimming performance and aerobic metabolism for the juvenile rainbow trout exposed to dietary SeMet.

In addition, temperature can influence the resting metabolism of fishes, potentially influencing oxygen consumption under times of stress. A positive relationship between resting metabolic rate and temperature exists in fish (Johnston et al., 1991). Clarke and Johnston (1999) reported that a tropical fish at 30°C require approximately six times as much oxygen for resting metabolism as does a polar fish at 0°C . This was reflected in the different SMR values measured for both fathead minnow and rainbow trout in this thesis as fathead minnow had greater SMR than rainbow trout. In addition, temperature and ecological lifestyle are two key factors that can influence the aerobic metabolism in fishes (Clarke and Pörtner, 2010). The resting metabolic rate can

be regarded as the energetic cost of temperature adaptation and is an integration of the costs, benefits and ecological lifestyle (Clarke and Fraser, 2004). Teleost fish data show that species that have higher resting metabolic rates when compared across taxa at similar temperatures have more active lifestyles (Zimmermann and Hubold, 1998; Clarke and Pörtner, 2010). Perhaps the higher SMR of juvenile fathead minnow in the present study was a result of a naturally active ecological lifestyle, and thus became negatively affected by the limited oxygen availability at 23°C during the swim performance testing.

Interestingly, rainbow trout could also be classified as having an active ecological lifestyle. They are a high performance fish and are known for swimming great distances upstream against substantial currents and jumping up waterfalls during reproductive spawning and migration (Milligan, 1996). It is possible that this naturally inherent swimming ability allowed the juvenile rainbow trout to be more tolerant to the effects of dietary SeMet exposure on aerobic metabolism associated with forced swimming tests compared to juvenile fathead minnow.

Similar to the fathead minnow results from this thesis, studies involving dietary SeMet exposure in the warm water zebrafish species conducted at 28°C reported impaired swimming performance along with increased MO_2 , COT, SMR (Thomas et al., 2013). In addition, rainbow trout acclimated to cold temperatures were unwilling or unable to swim continuously for extended periods (Kieffer et al., 1998), potentially impacting aerobic metabolism. Temperature can also place limitations on muscle performance in fish. Comparative studies of white glycolytic muscle of fish have shown that muscle power output increases strongly with temperature (Clarke and Pörtner, 2010). Thus, it could be possible that impaired swim performance in juvenile rainbow trout in the present study

could have been influenced by decreased muscle output as opposed to impaired aerobic metabolism. The results of the two experiments with respect to swim performance and aerobic metabolism suggest that juvenile fathead minnow were more sensitive to the effects of dietary SeMet exposure than juvenile rainbow trout. These differences may be due to exposure temperatures, ecological lifestyles, and species sensitivity differences. However, further investigation is required to fully understand the effects of dietary SeMet exposure on the swimming performance and oxygen consumption of juvenile fish species at different temperatures.

Future research to better characterize the effects of elevated dietary SeMet exposure on swim performance and aerobic metabolism in juvenile fishes could include a number of approaches. There are alternative swimming endpoints to U_{crit} that warrant investigation. For example, in back-to-back or repeat U_{crit} tests fish are subjected to two successive U_{crit} tests separated by a short recovery period. Repeat swim performance is considered a more sensitive measure of fish health and is often used to investigate recovery rates of fish after exposure to stressors (Jain et al., 1998). Adult zebrafish have shown impaired repeat swim performance and altered oxygen consumption in response to dietary SeMet exposure and aqueous selenite exposure (Massé et al., 2013; Thomas et al., 2013). Therefore, repeat U_{crit} testing may provide a more ecologically relevant measure of swimming and metabolism by integrating recovery rates of fish into swim performance testing.

Other swimming performance tests to consider also include sustained or burst swimming tests as discussed in Chapter 1 of this thesis. Burst tests primarily measure anaerobic capacity and it would be of interest to further characterize the storage and

anaerobic catabolism of glycogen during swimming. In particular, burst swimming tests could be of interest given that fathead minnow are typically a schooling, non-migratory fish that may depend on burst swimming activity to avoid predators.

Another alternative swimming test to U_{crit} is the measurement of the gait transition speed, which is the transition from steady cruising to burst-and-glide swimming (Videler, 1993). During fish cruising mode, swimming is powered by aerobic muscle action, while burst-and-glide swimming is powered by anaerobic muscle action (Videler, 1993). It is characterized by large and discrete upstream motion, increased tail-beat amplitude and increased tail-beat frequency (Tudorache et al., 2007, 2010). In this thesis, juvenile fathead minnow experienced decreased tail-beat amplitude and decreased tail-beat frequency in response to dietary SeMet exposure. Therefore, gait transition warrants further investigation and could provide an ecologically and physiologically relevant endpoint because it can assess both aerobic and anaerobic metabolism of fish swimming.

Further studies could also investigate the influence of exposure water temperature on the effects of dietary SeMet exposure in fish. Because water temperature is inversely related to the amount of dissolved oxygen, exposing the same fish species at a range of temperatures may help to characterize the influence of temperature on effects of elevated dietary SeMet exposure on swimming performance and aerobic metabolism. For example, fathead minnow are tolerant of a number of water quality conditions including temperature, and would make an attractive species for this investigation. Furthermore, in order to help identify further species and life stage sensitivity differences in the effects of dietary SeMet exposure, experiments using identical dietary exposure protocols could be carried out in various life stages and at different temperatures in the same fish species.

4.2.4 Bioenergetics and physiological stress response

Triglycerides and glycogen are the key forms of stored energy in fish and were used to investigate energy storage capacity and metabolism in this thesis. Whole body triglyceride concentrations in fathead minnow were greater in the 5.4 $\mu\text{g Se/g}$ group compared to controls and were decreased in swam fish versus non-swam fish in the same treatment group. Whole body glycogen concentrations were decreased in all non-swam SeMet exposure groups while the 5.4 and 26.5 $\mu\text{g Se/g}$ exposure groups had increased glycogen concentrations in swam versus non-swam fathead minnow. Whole body concentrations of energy stores were necessary for juvenile fathead minnows because it was not feasible to use liver and muscle tissue due to their small body size. Liver and muscle concentrations were used for juvenile rainbow trout as their mass was sufficient enough upon completion of the dietary SeMet exposure. Unlike fathead minnow, dietary SeMet exposure did not have any significant effects on muscle triglycerides in rainbow trout. The swim trial did result in decreased liver and muscle triglycerides in rainbow trout. Interestingly, while dietary SeMet exposure increased whole body triglyceride concentrations in fathead minnow, the liver triglyceride concentrations were decreased in both swam and non-swam rainbow trout. Similar to fathead minnow, muscle glycogen was decreased in exposed non-swam trout compared to controls and also elevated in swam fish compared to non-swam fish in respective treatment groups. Liver glycogen in juvenile rainbow trout was largely unaffected by dietary SeMet exposure and swim status.

The results presented above demonstrate the importance of triglycerides during aerobic swimming. Triglyceride concentrations were decreased in both fathead minnow

and rainbow trout after swimming, suggesting that they were used to fuel swimming. Indeed, critical swimming speed is a measure of aerobic swimming and is fuelled primarily by intramuscular triglyceride stores (Moyes and West, 1995; Tocher, 2003). Elevated glycogen stores in swam fish in both experiments also point towards triglycerides as the main fuel source for swimming. During U_{crit} testing, anaerobic glycolysis is the energy source for swimming beyond 80% of U_{crit} (Webb, 1971). The mobilization of glycogen is an important part of the acute stress response, and increased glycogen stores in swam fish could indicate some impairment. Indeed, an attenuation of the cortisol response has been shown in rainbow trout exposed to dietary SeMet (Wiseman et al., 2011b). Interestingly, whole body cortisol concentrations in the present thesis were increased with swimming in fathead minnow and unaffected by SeMet concentration in rainbow trout. Further investigation is required to determine the complex relationship between the physiological stress response, energy homeostasis and swimming performance in juvenile fish species.

Previous studies investigating critical swim speed and dietary SeMet exposure demonstrated a dose dependent increase in triglyceride and glycogen stores with increasing dietary Se concentration in zebrafish (Thomas and Janz, 2011; Thomas et al., 2013). It was anticipated that similar trends would emerge in juvenile fathead minnow and rainbow trout, however this was not the case. The absence of dose dependent increases in energy stores may indicate species and life stage sensitivity differences compared to zebrafish. In addition, other intermediary metabolic endpoints have been affected by Se exposure. Adult zebrafish exposed to dietary SeMet had down regulation of β -hydroxyacyl coenzyme A dehydrogenase (HOAD), a key enzyme involved in

triglyceride catabolism (Thomas et al., 2013). In the same study by Thomas et al. (2013), an increase in whole body lactate was also observed. Lactate is a by-product of anaerobic energy catabolism and can be measured to assess anaerobic metabolic activity due to its accumulation in muscle tissue during burst swimming. Furthermore, citrate synthase is a rate-limiting enzyme in the citric acid cycle and can be used as an indicator of tissue aerobic scope. Decreased citrate synthase activity has been demonstrated in juvenile fathead minnow exposed to Se-impacted site water (Goertzen et al., 2011). Future studies should consider the use of endpoints of intermediary metabolism in fish to gain a more comprehensive understanding of how dietary SeMet exposure affects the energy storage capacity and usage in juvenile fish species.

The effects of temperature on energy metabolism and energy storage capacity must be considered when evaluating the effect of dietary SeMet exposure in this thesis. As discussed above, exposure temperature may influence aerobic metabolism when it comes to swimming performance in juvenile fathead minnow and rainbow trout. Thus, some effects may occur in related physiological processes such as altered energy stores and cortisol secretion. Indeed, it has been reported that there is an energetic advantage to fish inhabiting cold water systems as a result of decreased maintenance costs (Clarke and Johnston, 1999). Because a lower resting metabolic rate is associated with fish at lower temperatures, it follows that perhaps less energy is required for physiological and detoxification processes. Therefore, due to the lower energetic cost at low temperature, juvenile rainbow trout were less affected by the energetic demands of dietary SeMet exposure than juvenile fathead minnow in this thesis and adult zebrafish in other studies. Future research should focus on the energetic storage response at a range of temperatures

in a range of species and life stages to better characterize the effects of dietary SeMet exposure on juvenile fish.

4.3 Conclusions

The present thesis investigated sublethal effects of dietary Se exposure on swimming performance, aerobic metabolism, energy homeostasis and the physiological stress response in two juvenile fish species. Swimming performance was negatively impacted by dietary SeMet exposure in both juvenile fathead minnow and juvenile rainbow trout. Impaired muscle function may be one possible cause of impaired swimming performance. Additionally, altered aerobic metabolism in juvenile fathead minnow may have led to the decrease in U_{crit} . Interestingly, endpoints measured during the swim challenge to assess aerobic metabolism were not significantly affected by dietary SeMet exposure in juvenile rainbow trout, suggesting possible species differences. Compared to the published literature, altered energy homeostasis in both juvenile fathead minnow and rainbow trout in response to dietary SeMet may indicate potential species and life stage sensitivity differences. The sublethal responses observed in this thesis were caused by whole body burdens of Se similar to thresholds previously reported in eggs, suggesting that these are among the most sensitive endpoints in response to direct dietary SeMet exposure. Impaired swim performance may increase predation risk, alter food acquisition and migration of fish inhabiting aquatic ecosystems contaminated with Se, thus adversely impacting fitness and survivability. It remains to be seen if these sublethal effects in juvenile fish will negatively influence population dynamics in natural habitats.

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